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-27                                  1
 MDLR AGDSWGMLAC LCTVLWHLPA VPALNRTGDP GPGPSIQKTY    17

DLTRYLEHQL RSLAGTYLNY LGPPFNEPDF NPPRLGAETL PRATVDLEVW    67

RSLNDKLRLT QNYEAYSHLL CYLRGLNRQA ATAELRRSLA HFCTSLQGLL   117

GSIAGVMAAL GYPLPQPLPG TEPTWTPGPA HSDFLQKMDD FWLLKELQTW   167

                                    198
LWRSAKDFNR LKKKMQPPAA AVTLHLGAHG F*                      198
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(57) Abstract

Disclosed are nucleic acids encoding novel neurotrophic factors, designated NNT–1. Also disclosed are amino acid sequences for NNT–1 polypeptides, methods for preparing NNT–1 polypeptides, and other related aspects. Such polypeptides are active in stimulating B–cell and/or T cell production, as well as reducing inflammatory responses.

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THE NEUROTROPHIC FACTOR NNT-1

This application is a continuation-in-part of application Serial No. 08/792,019 filed February 3, 1997 which is hereby incorporated by reference.

BACKGROUND

Field of the Invention

This invention relates to a novel polypeptide designated NNT-1 and related polypeptides that have neurotrophic activity, to novel nucleic acid molecules encoding such polypeptides, and to other related aspects.

Description of Related Art

A number of neurological disorders and diseases are caused at least in part by degeneration or death of particular classes of neurons. For example, Parkinson's disease is characterized by slowing of voluntary muscle movement, muscular rigidity, and tremor. Such symptoms are attributed at least in part to progressive degeneration of dopamine-producing neurons located in a specific region of the brain called the substantia nigra. Degeneration of these neurons ("dopaminergic neurons") results in a decrease of dopamine levels in an adjacent region of the brain called the striatum. The striatum contains neurons expressing receptors for dopamine; these neurons are involved in the control of motor activity. The cause of the degeneration of dopaminergic neurons is unknown, but has been attributed to free radicals, excess iron content, environmental toxins, excitatory amino acid neurotoxicity, and possibly a deficiency of certain neurotrophic factors (Jenner, *Neurology*, Suppl. 3:S6-

S12 [1995]; Adams and Victor, eds. *Principles of Neurology*, Chapter 42: Degenerative Diseases of the Nervous System, McGraw Hill, NY [1993]).

Diseases such as amyotrophic lateral sclerosis (ALS; also known as Lou Gehrig's disease), progressive muscular atrophy, and hereditary motor and sensory neuropathy (Charcot-Marie-Tooth disease) all result at least in part from a decay of motor neurons which are located in the ventral horn of the spinal cord.

The hippocampus, a well defined structure that is part of the cerebral cortex of the brain, is important in the formation of long term memory. Destruction of the hippocampus, for example by ischemia, can result in an inability to form new memories. Degeneration of pyramidal CA1 neurons, which are located in the CA1 region of the hippocampus, is one characteristic of Alzheimer's disease. These same neurons are selectively vulnerable to ischemic and anoxic damage which occur in conditions such as stroke and head trauma. In addition, the CA1 pyramidal hippocampal neurons as well as pyramidal neurons located in the CA3 region of the hippocampus, are selectively injured in epilepsy.

The striatum is the innervation region of the nerve terminals of dopaminergic-containing neurons from the substantia nigra. The majority of striatal neurons utilize GABA (4-aminobutyric acid) as their neurotransmitter. The striatum is the major target of the progressive neurodegeneration that occurs in Huntington's disease, in which the major neuron loss is that of the striatal GABA-utilizing neurons.

The serotonin-containing neurons are located in groups clustered around the midline of the hindbrain. These neurons are involved in the control of body temperature, mood, and sleep. Disorders of the

serotonin-containing neuron system include, for example, depression, other mood disorders, and sleep disturbances.

Photoreceptor cells are a specialized subset of retina neurons, and are responsible for vision. Injury and/or death of photoreceptor cells can lead to blindness. Degeneration of the retina, such as by retinitis pigmentosa, age-related macular degeneration, and stationary night blindness, are all characterized by the progressive atrophy and loss of function of photoreceptor outer segments which are specialized structures containing the visual pigments that transform a light stimulus into electrical activity.

While there are some therapies available to treat the symptoms and decrease the severity of such diseases (*e.g.*, L-dopa to treat Parkinson's disease), there currently exists no effective treatment to prevent or reduce the degeneration of most of the above mentioned classes of affected neurons, or to promote their repair.

Recently, several naturally occurring proteinaceous molecules have been identified based on their trophic activity on various neurons. These molecules are termed "neurotrophic factors". Neurotrophic factors are endogenous, soluble proteins that can stimulate or regulate survival, growth, and/or morphological plasticity of neurons (see Fallon and Laughlin, *Neurotrophic Factors*, Academic Press, San Diego, CA [1993]).

The known neurotrophic factors belong to several different protein superfamilies of polypeptide growth factors based on their amino acid sequence homology and/or their three-dimensional structure (MacDonald and Hendrikson, *Cell*, 73:421-424 [1993]). One family of neurotrophic factors is the neurotrophin family. This family currently consists of NGF (nerve

growth factor), BDNF (brain derived neurotrophic factor), NT-3 (neurotrophin-3), NT-4 (neurotrophin-4), and NT-6 (neurotrophin-6).

CNTF (ciliary neurotrophic factor) and LIF (leukemia inhibitory factor) are cytokine polypeptides that have neurotrophic activity. By virtue of their structural features and receptor components, these polypeptides are related to a family of hematopoietic cytokines that includes IL-6 (interleukin-6), IL-11 (interleukin-11), G-CSF (granulocyte-colony stimulating factor), and oncostatin-M. NNT-1 of the present invention exhibits significant similarity to various members of this family of neurotrophic factors. See FIG. 6.

GDNF (glial derived neurotrophic factor) is a neurotrophic factor that belongs to the TGF-beta (transforming growth factor beta) superfamily. GDNF displays potent survival and differentiation-promoting actions for dopaminergic and motor neurons (Lin *et al.*, *Science*, 260:1130-1132 [1993]; Yan *et al.*, *Nature*, 373:341-344 [1995]).

While these neurotrophic factors are known to increase growth and/or survival of neurons, there is less known about the molecules that work in conjunction with these factors. One manner in which additional neurotrophins and related molecules may be identified is to administer to an animal one or more compounds known to have an effect on the nervous system, and to then analyze tissues for the induction of genes involved in neural responses to the compounds. For example, one can screen for genes that are induced in certain tissues of the nervous system, such as the hippocampal region of the brain. This technique was used by Nedivi *et al* (*Nature*, 363:718-722 [1993]; Nedivi et al., *Proc. Natl. Acad. Sci USA*, 93:2048-2053 [1996]) to identify novel genes that are induced in the

dentate gyrus portion of the hippocampus in response to administration of a neurotransmitter analog of glutamate called kainate (kainic acid).

Expression of many neurotrophic factors such as NGF, BDNF, NT3, GDNF, bFGF, IGF-1 and TGF-beta is regulated by afferent neuronal activity and/or by neuronal injury. Strong induction of some of these genes can be observed in the hippocampus dentate gyrus in response to the glutamate analog kainate (Isackson, *Current Opinions in Neurobiology* 5:50-357 [1995]). Kainate treatment appears to increase the release of novel compounds from the hippocampus of alert rats, and this activity appears to be different from the actions of known neurotrophic factors (Humpel, *et al., Science,* 269:552-554 [1995]).

In view of the fact that many nervous system disorders and diseases have no known cure, there is a need in the art to identify novel compounds for treating neurological conditions and diseases such as Parkinson's disease, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, stroke, and various degenerative disorders that affect vision.

There is additional evidence presented herein that NNT-1 compounds may have a biological activity of modulating the immune system, in particular by causing an increase in B-cell and T-cell production.

Accordingly, it is an object of the present invention to provide novel compounds that may be useful in promoting neuron regeneration and restoring neural functions.

It is a further object of the invention to provide a method of treating neurological diseases such as those set forth herein.

It is still a further object of the invention to provide a method of treating immunological diseases such as those set forth herein.

These and other objects will be apparent to one of ordinary skill in the art from the present disclosure.

SUMMARY OF THE INVENTION

In one embodiment, the present invention provides a nucleic acid molecule encoding a polypeptide selected from the group consisting of:

(a) the nucleic acid molecule of SEQ ID NO:1;

(b) the nucleic acid molecule of SEQ ID NO:3;

(c) a nucleic acid molecule encoding the polypeptide of SEQ ID NO:2 or a biologically active fragment thereof;

(d) a nucleic acid molecule that encodes a polypeptide that is at least 70 percent identical to the polypeptide of SEQ ID NO:2;

(e) a nucleic acid molecule that hybridizes under stringent conditions to any of (a)-(d) above; and

(f) a nucleic acid molecule that is the complement of any of (a)-(e) above.

In another embodiment, the present invention provides a nucleic acid molecule encoding a polypeptide selected from the group consisting of:

(a') the nucleic acid molecule of SEQ ID NO:4;

(b') a nucleic acid molecule encoding the polypeptide of SEQ ID NO:5 or a biologically active fragment thereof;

(c') a nucleic acid molecule that encodes a polypeptide that is at least 70 percent identical to the polypeptide of SEQ ID NO:5;

(d') a nucleic acid molecule that hybridizes under stringent conditions to any of (a')-(c') above; and

(e') a nucleic acid molecule that is the complement of any of (a')-(d') above.

In another embodiment, the invention provides vectors comprising these nucleic acid molecules, and host cells, either prokaryotic or eukaryotic, comprising the vectors.

The invention further provides an NNT-1 polypeptide selected from the group consisting of:

(a) the polypeptide of SEQ ID NO:2;

(b) the polypeptide that is amino acids 1-198 of SEQ ID NO:2;

(c) a polypeptide that is at least 70 percent identical to the polypeptide of (a) or (b); and

(d) a biologically active fragment of any of (a)-(c).

The invention further provides an NNT-1 polypeptide selected from the group consisting of:

(a') the polypeptide of SEQ ID NO:5;

(b') the polypeptide that is amino acids 1-198 of SEQ ID NO:5;

(c') a polypeptide that is at least 70 percent identical to the polypeptide of (a') or (b'); and

(d') a biologically active fragment of any of (a')-(c').

Optionally, the NNT-1 polypeptide may or may not have an amino terminal methionine.

In another embodiment, the invention provides a process for producing an NNT-1 polypeptide, wherein the polypeptide may be SEQ ID NO:2 or SEQ ID NO:5, amino acids 1-198 of SEQ ID NO:2, amino acids 1-198 of SEQ ID NO: 5, or a biologically active fragment thereof, and wherein the process comprises:

(a) expressing a polypeptide encoded by an NNT-1 nucleic acid molecule in a suitable host; and

(b) isolating the polypeptide.

The invention further provides anti-NNT-1 antibodies.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the nucleic acid sequence of the cDNA encoding human NNT-1 (SEQ ID NO:1).

Figure 2 depicts the nucleic acid sequence of the human genomic DNA for NNT-1 (SEQ ID NO:3).

Figure 3 depicts the amino acid sequence for human NNT-1 (SEQ ID NO:1) as translated from the cDNA (SEQ ID NO:2). The first 27 amino acids may represent a signal peptide sequence, such that the mature form of NNT-1 starts at the leucine indicated as number 1. The * indicates the stop codon.

Figure 4 depicts the nucleic acid sequence of the cDNA encoding murine NNT-1 (SEQ ID NO:4).

Figure 5 depicts the amino acid sequence for murine NNT-1 (SEQ ID NO:5) as translated from the cDNA (SEQ ID NO:4). The first 27 amino acids may represent a signal peptide sequence, such that the mature form of murine NNT-1 starts at the leucine indicated as number 1. The * indicates the stop codon.

Figure 6 depicts a comparison of amino acid sequences of NNT-1, IL-11 (SEQ ID NO:8), IL-6 (SEQ ID NO:9), G-CSF (SEQ ID NO:10), cardiotrophin (SEQ ID NO:11), CNTF (SEQ ID NO:12), oncostatin (SEQ ID NO:13), and LIF (SEQ ID NO:14). In each case, the human molecule is compared.

Figure 7 depicts a graph of the results of a chick motor neuron activity assay for human NNT-1 compared to human CNTF.

Figure 8 depicts a graph of the results of a chick sympathetic neuron activity assay for human NNT-1 compared to human CNTF.

Figure 9 depicts a normal spleen from a negative control mouse (#22), 20x objective, H&E stain.

Figure 10 depicts a spleen from an NNT-1 transgenic mouse (#62) with lymphoid hyperplasia (arrow).

Figure 11 depicts a normal liver from a control mouse, 10x objective, H&E stain.

Figure 12 depicts a liver from an NNT-1 transgenic mouse (#60) with lymphoid aggregates in sinusoids (arrow) and around vessels, H&E stain.

Figure 13 depicts data showing that NNT-1 induced serum SAA ($p<0.001$). There were five mice per group.

Figure 14 depicts data showing that NNT-1 potentiated the induction by IL-1 of corticosterone in serum ($p < 0.01$) and increased serum levels of corticosterone also independently of IL-1 ($p < 0.001$). There were five mice per group.

Figure 15 depicts data showing that NNT-1 potentiated the induction by IL-1 of IL-6 in serum ($p < 0.001$). There were five mice per group.

Figure 16 depicts data showing that NNT-1 blocked the LPS-induced increased of serum TNF levels ($p < 0.001$). There were ten mice in the LPS-treated groups, five in the others.

Figure 17 depicts data showing that NNT-1 increased the counts of total ($p < 0.04$) and CD45-positive cells in peripheral lymph nodes in mice ($p < 0.001$).

DETAILED DESCRIPTION OF THE INVENTION

Included in the scope of this invention are NNT-1 polypeptides such as the polypeptides of SEQ ID NO:2 or SEQ ID NO: 5, and related biologically active polypeptide fragments and derivatives thereof. Further included within the scope of the present invention are nucleic acid molecules that encode these polypeptides, and methods for preparing the polypeptides.

I. NNT-1 Proteins/Polypeptides, Fragments and Derivatives Thereof

The term "NNT-1 protein" or "NNT-1 polypeptide" as used herein refers to any protein or polypeptide having the properties described herein for NNT-1. The NNT-1 polypeptide may or may not have an amino terminal methionine, depending, for example, on the manner in which it is prepared. By way of illustration, NNT-1 protein or NNT-1 polypeptide refers to:

(1) an amino acid sequence encoded by NNT-1 nucleic acid molecules as defined in any of the following items:

(a) the nucleic acid molecule of SEQ ID NO:1;

(b) the nucleic acid molecule of SEQ ID NO:3;

(c) a nucleic acid molecule encoding the polypeptide of SEQ ID NO:2 or a biologically active fragment thereof;

(d) a nucleic acid molecule that encodes a polypeptide that is at least 70 percent identical to the polypeptide of SEQ ID NO:2;

(e) a nucleic acid molecule that hybridizes under stringent conditions to any of (a)-(d) above; and

(f) a nucleic acid molecule that is the complement of any of (a)-(e) above; and

(a') the nucleic acid molecule of SEQ ID NO:4;

(b') a nucleic acid molecule encoding the polypeptide of SEQ ID NO:5 or a biologically active fragment thereof;

(c') a nucleic acid molecule that encodes a polypeptide that is at least 70 percent identical to the polypeptide of SEQ ID NO:5;

(d') a nucleic acid molecule that hybridizes under stringent conditions to any of (a')-(c') above; and

(e') a nucleic acid molecule that is the complement of any of (a')-(d') above; and

(2) naturally occurring allelic variants of the NNT-1 gene which result in one or more amino acid substitutions, deletions, and/or insertions as compared to the NNT-1 polypeptide of SEQ ID NO:2 or SEQ ID NO: 5, and/or

(3) chemically modified derivatives as well as nucleic acid and or amino acid sequence variants thereof as provided for herein.

The NNT-1 polypeptides that have use in practicing the present invention may be naturally

occurring full length polypeptides, or truncated polypeptides or peptides (*i.e*, "fragments").

The polypeptides may be in mature form or they may be attached to a native or heterogeneous signal peptide. For example, human and murine NNT-1 have signal peptides of amino acids -27 to -1 of SEQ ID NOS: 2 and 5, respectively.

The polypeptides or fragments may be chemically modified, *i.e.*, glycosylated, phosphorylated, and/or linked to a polymer, as described below, and they may have an amino terminal methionine, depending on how they are prepared. In addition, the polypeptides or fragments may be variants of the naturally occurring NNT-1 polypeptide (*i.e.*, may contain one or more amino acid deletions, insertions, and/or substitutions as compared with naturally occurring NNT-1).

As used herein, the term "NNT-1 fragment" refers to a peptide or polypeptide that is less than the full length amino acid sequence of naturally occurring NNT-1 protein but has qualitatively a substantially similar type of biological activity as NNT-1 polypeptide or NNT-1 protein described above. Such a fragment may be truncated at the amino terminus, the carboxy terminus, or both, and may be chemically modified. Such NNT-1 fragments may be prepared with or without an amino terminal methionine. The activity of the fragments may be greater than, the same as, or less than the full-length (mature) NNT-1 polypeptide. Preferably, the activity of the fragment is $\geq$50%, more preferably $\geq$65%, most preferably $\geq$80%, of the activity of the full-length polypeptide, as measured by a standard activity assay, such as those set forth in the Examples section herein. Some exemplary fragments of this invention include the polypeptides wherein from 1 to 20 amino acids are removed from either the C-

terminus, the N-terminus, or both termini, of the NNT-1 polypeptide.

As used herein, the term "NNT-1 derivative" or "NNT-1 variant" refers to an NNT-1 polypeptide, protein, or fragment that 1) has been chemically modified, as for example, by addition of one or more polyethylene glycol molecules, sugars, phosphates, or other such molecules not naturally attached to wild-type NNT-1 polypeptide, and/or 2) contains one or more nucleic acid or amino acid sequence substitutions, deletions, and/or insertions as compared to the NNT-1 amino acid sequence set forth in Figure 3 (human) or Figure 5 (murine).

As used herein, the terms "biologically active polypeptide" and "biologically active fragment" refer to a peptide or polypeptide in accordance with the above description for NNT-1 wherein the NNT-1 acts as a growth factor for (a) neurons (e.g., motor neurons and/or sympathetic neurons) or (b) immunological cells, such as B cells and T cells.

Fragments and/or derivatives of NNT-1 that are not themselves active in activity assays may be useful as modulators (e.g., inhibitors or stimulants) of the NNT-1 receptors *in vitro* or *in vivo*, or to prepare antibodies to NNT-1 polypeptides.

The amino acid variants of NNT-1 of this invention preferably are at least 70% identical to either SEQ ID NO: 2 or SEQ ID NO: 5, more preferably at least about 80% identical, even more preferably at least about 90% identical.

Percent sequence identity can be determined by standard methods that are commonly used to compare the similarity in position of the amino acids of two polypeptides. By way of example, using a computer program such as BLAST or FASTA, the two polypeptides for which the percent sequence identity is to be

determined are aligned for optimal matching of their respective amino acids (the "matched span", which can include the full length of one or both sequences, or a predetermined portion of one or both sequences). Each computer program provides a "default" opening penalty and a "default" gap penalty, and a scoring matrix such as PAM 250. A standard scoring matrix (see Dayhoff *et al.*, in: *Atlas of Protein Sequence and Structure*, vol. 5, supp.3 [1978]) can be used in conjunction with the computer program. The percent identity can then be calculated using an algorithm contained in a program such as FASTA as:

$$\frac{\text{Total number of identical matches}}{\begin{array}{l}\text{[length of the longer sequence within the matched span] +} \\ \text{[number of gaps introduced into the longer sequence in order to align the two sequences]}\end{array}} \times 100$$

Polypeptides that are at least 70 percent identical will typically have one or more amino acid substitutions, deletions, and/or insertions as compared with wild type NNT-1. Usually, the substitutions will be conservative so as to have little or no effect on the overall net charge, polarity, or hydrophobicity of the protein but optionally may increase the activity of NNT-1. Conservative substitutions are set forth in Table I below.

Table I

Conservative amino acid substitutions

Basic:	arginine
	lysine
	histidine
Acidic:	glutamic acid
	aspartic acid

Polar:	glutamine
	asparagine
Hydrophobic:	leucine
	isoleucine
	valine
Aromatic:	phenylalanine
	tryptophan
	tyrosine
Small:	glycine
	alanine
	serine
	threonine
	methionine

The invention also encompasses species homologs of NNT-1; for example, NNT-1 homologs from a mammalian species such as dog, cat, mouse, rat, monkey, horse, pig, goat, rabbit, sheep and the like is contemplated in addition to human. The sequences of murine cDNA and protein are provided as SEQ ID NOS: 4 and 5.

The invention further encompasses chimeric polypeptides, such as NNT-1 attached to all or a portion of another polypeptide. Preferably the chimeric polypeptide comprises NNT-1 attached to all or a portion of another neurotrophic factor, such as BDNF, GDNF, NT-3, NT-4, NT-5, NT-6, and the like. The polypeptides may be attached N to C terminus, C to C terminus, or N to N terminus.

II. Nucleic Acids

As used herein, the term "NNT-1" when used to describe a nucleic acid molecule refers to a nucleic acid molecule or fragment thereof, as set forth above.

The term "stringent conditions" refers to hybridization and washing under conditions that permit

only binding of a nucleic acid molecule such as an oligonucleotide or cDNA molecule probe to highly homologous sequences. One stringent wash solution is 0.015 M NaCl, 0.005 M NaCitrate, and 0.1 percent SDS used at a temperature of 55°C-65°C. Another stringent wash solution is 0.2 X SSC and 0.1 percent SDS used at a temperature of between 50°C-65°C. Where oligonucleotide probes are used to screen cDNA or genomic libraries, the following stringent washing conditions may be used. One protocol uses 6 X SSC with 0.05 percent sodium pyrophosphate at a temperature of 35°C-62°C, depending on the length of the oligonucleotide probe. For example, 14 base pair probes are washed at 35-40°C, 17 base pair probes are washed at 45-50°C, 20 base pair probes are washed at 52-57°C, and 23 base pair probes are washed at 57-63°C. The temperature can be increased 2-3°C where the background non-specific binding appears high. A second protocol utilizes tetramethylammonium chloride (TMAC) for washing oligonucleotide probes. One stringent washing solution is 3 M TMAC, 50 mM Tris-HCl, pH 8.0, and 0.2 percent SDS. The washing temperature using this solution is a function of the length of the probe. For example, a 17 base pair probe is washed at about 45-50°C.

NNT-1 nucleic acid molecules, fragments, and/or derivatives that do not themselves encode polypeptides that are active in activity assays may be useful as hybridization probes in diagnostic assays to test, either qualitatively or quantitatively, for the presence of NNT-1 DNA or RNA in mammalian tissue or bodily fluid samples.

NNT-1 nucleic acid molecules encoding NNT-1 polypeptides attached to native or heterogeneous signal peptides and/or to chimeric polypeptides as described

herein above are also included within the scope of this invention.

III. Methods for Preparing NNT-1 Polypeptides

A. Recombinant Methods

The full length NNT-1 polypeptide or fragment thereof can be prepared using well known recombinant DNA technology methods such as those set forth in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY [1989]) and/or Ausubel *et al.*, eds, (*Current Protocols in Molecular Biology*, Green Publishers Inc. and Wiley and Sons, NY [1994]). A gene or cDNA encoding the NNT-1 protein or fragment thereof may be obtained for example by screening a genomic or cDNA library, or by PCR amplification. Alternatively, a gene encoding the NNT-1 polypeptide or fragment may be prepared by chemical synthesis using methods well known to the skilled artisan such as those described by Engels *et al.* (*Angew. Chem. Intl. Ed.*, 28:716-734 [1989]). These methods include, inter alia, the phosphotriester, phosphoramidite, and H-phosphonate methods for nucleic acid synthesis. A preferred method for such chemical synthesis is polymer-supported synthesis using standard phosphoramidite chemistry. Typically, the DNA encoding the NNT-1 polypeptide will be several hundred nucleotides in length. Nucleic acids larger than about 100 nucleotides can be synthesized as several fragments using these methods. The fragments can then be ligated together to form the full length NNT-1 polypeptide. Usually, the DNA fragment encoding the amino terminus of the polypeptide will have an ATG, which encodes a methionine residue. This methionine may or may not be present on the mature form of the NNT-1 polypeptide, depending on whether the

polypeptide produced in the host cell is secreted from that cell.

In some cases, it may be desirable to prepare nucleic acid and/or amino acid variants of naturally occurring NNT-1. Nucleic acid variants (wherein one or more nucleotides are designed to differ from the wild-type or naturally occurring NNT-1) may be produced using site directed mutagenesis or PCR amplification where the primer(s) have the desired point mutations (see Sambrook *et al.*, *supra*, and Ausubel *et al.*, *supra*, for descriptions of mutagenesis techniques). Chemical synthesis using methods described by Engels *et al.*, *supra*, may also be used to prepare such variants. Other methods known to the skilled artisan may be used as well. Preferred nucleic acid variants are those containing nucleotide substitutions accounting for codon preference in the host cell that is to be used to produce NNT-1. Other preferred variants are those encoding conservative amino acid changes as described above (*e.g.*, wherein the charge or polarity of the naturally occurring amino acid side chain is not altered substantially by substitution with a different amino acid) as compared to wild type, and/or those designed to either generate a novel glycosylation and/or phosphorylation site(s) on NNT-1, or those designed to delete an existing glycosylation and/or phosphorylation site(s) on NNT-1.

The NNT-1 gene or cDNA can be inserted into an appropriate expression vector for expression in a host cell. The vector is selected to be functional in the particular host cell employed (*i.e.*, the vector is compatible with the host cell machinery such that amplification of the NNT-1 gene and/or expression of the gene can occur). The NNT-1 polypeptide or fragment thereof may be amplified/expressed in prokaryotic, yeast, insect (baculovirus systems) and/or eukaryotic

host cells. Selection of the host cell will depend at least in part on whether the NNT-1 polypeptide or fragment thereof is to be glycosylated. If so, yeast, insect, or mammalian host cells are preferable; yeast cells will glycosylate the polypeptide, and insect and mammalian cells can glycosylate and/or phosphorylate the polypeptide as it naturally occurs on the NNT-1 polypeptide (*i.e.*, "native" glycosylation and/or phosphorylation).

Typically, the vectors used in any of the host cells will contain 5' flanking sequence (also referred to as a "promoter") and other regulatory elements as well such as an enhancer(s), an origin of replication element, a transcriptional termination element, a complete intron sequence containing a donor and acceptor splice site, a signal peptide sequence, a ribosome binding site element, a polyadenylation sequence, a polylinker region for inserting the nucleic acid encoding the polypeptide to be expressed, and a selectable marker element. Each of these elements is discussed below. Optionally, the vector may contain a "tag" sequence, *i.e.*, an oligonucleotide sequence located at the 5' or 3' end of the NNT-1 coding sequence that encodes polyHis (such as hexaHis) or another small immunogenic sequence. This tag will be expressed along with the protein, and can serve as an affinity tag for purification of the NNT-1 polypeptide from the host cell. Optionally, the tag can subsequently be removed from the purified NNT-1 polypeptide by various means such as using a selected peptidase for example.

The 5' flanking sequence may be homologous (*i.e.*, from the same species and/or strain as the host cell), heterologous (*i.e.*, from a species other than the host cell species or strain), hybrid (*i.e.*, a combination of 5' flanking sequences from more than one

source), synthetic, or it may be the native NNT-1 5' flanking sequence. As such, the source of the 5' flanking sequence may be any unicellular prokaryotic or eukaryotic organism, any vertebrate or invertebrate organism, or any plant, provided that the 5' flanking sequence is functional in, and can be activated by, the host cell machinery.

The 5' flanking sequences useful in the vectors of this invention may be obtained by any of several methods well known in the art. Typically, 5' flanking sequences useful herein other than the NNT-1 5' flanking sequence will have been previously identified by mapping and/or by restriction endonuclease digestion and can thus be isolated from the proper tissue source using the appropriate restriction endonucleases. In some cases, the full nucleotide sequence of the 5' flanking sequence may be known. Here, the 5' flanking sequence may be synthesized using the methods described above for nucleic acid synthesis or cloning.

Where all or only a portion of the 5' flanking sequence is known, it may be obtained using PCR and/or by screening a genomic library with suitable oligonucleotide and/or 5' flanking sequence fragments from the same or another species.

Where the 5' flanking sequence is not known, a fragment of DNA containing a 5' flanking sequence may be isolated from a larger piece of DNA that may contain, for example, a coding sequence or even another gene or genes. Isolation may be accomplished by restriction endonuclease digestion using one or more carefully selected enzymes to isolate the proper DNA fragment. After digestion, the desired fragment may be isolated by agarose gel purification, Qiagen® column or other methods known to the skilled artisan. Selection

of suitable enzymes to accomplish this purpose will be readily apparent to one of ordinary skill in the art.

The origin of replication element is typically a part of prokaryotic expression vectors purchased commercially, and aids in the amplification of the vector in a host cell. Amplification of the vector to a certain copy number can, in some cases, be important for optimal expression of the NNT-1 polypeptide. If the vector of choice does not contain an origin of replication site, one may be chemically synthesized based on a known sequence, and ligated into the vector.

The transcription termination element is typically located 3' of the end of the NNT-1 polypeptide coding sequence and serves to terminate transcription of the NNT-1 polypeptide. Usually, the transcription termination element in prokaryotic cells is a G-C rich fragment followed by a poly T sequence. While the element is easily cloned from a library or even purchased commercially as part of a vector, it can also be readily synthesized using methods for nucleic acid synthesis such as those described above.

A selectable marker gene element encodes a protein necessary for the survival and growth of a host cell grown in a selective culture medium. Typical selection marker genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, tetracycline, or kanamycin for prokaryotic host cells, (b) complement auxotrophic deficiencies of the cell; or (c) supply critical nutrients not available from complex media. Preferred selectable markers are the kanamycin resistance gene, the ampicillin resistance gene, and the tetracycline resistance gene.

The ribosome binding element, commonly called the Shine-Dalgarno sequence (prokaryotes) or the Kozak

sequence (eukaryotes), is necessary for translation initiation of mRNA. The element is typically located 3' to the promoter and 5' to the coding sequence of the NNT-1 polypeptide to be synthesized. The Shine-Dalgarno sequence is varied but is typically a polypurine (*i.e.*, having a high A-G content). Many Shine-Dalgarno sequences have been identified, each of which can be readily synthesized using methods set forth above and used in a prokaryotic vector.

In those cases where it is desirable for NNT-1 to be secreted from the host cell, a signal sequence may be used to direct the NNT-1 polypeptide out of the host cell where it is synthesized, and the carboxy-terminal part of the protein may be deleted in order to prevent membrane anchoring. Typically, the signal sequence is positioned in the coding region of NNT-1 nucleic acid sequence, or directly at the 5' end of the NNT-1 coding region. Many signal sequences have been identified, and any of them that are functional in the selected host cell may be used in conjunction with the NNT-1 gene. Therefore, the signal sequence may be homologous or heterologous to the NNT-1 polypeptide, and may be homologous or heterologous to the NNT-1 polypeptide. Additionally, the signal sequence may be chemically synthesized using methods set forth above. In most cases, secretion of the polypeptide from the host cell via the presence of a signal peptide will result in the removal of the amino terminal methionine from the polypeptide. Examples of secretory sequences useful for carrying out expression and secretion of NNT-1 polypeptides are selected from tPA leader sequences (see, e.g., Rickles et al., *J. Biol. Chem.* 263: 1563-1560 [1988] and Feng et al., *J. Biol. Chem.* 265: 2022-2027 [1990], EPO leader sequences and cardiotrophin leader sequences.

In many cases, transcription of the NNT-1 polypeptide is increased by the presence of one or more introns on the vector; this is particularly true where NNT-1 is produced in eukaryotic host cells, especially mammalian host cells. The introns used may be naturally occurring within the NNT-1 nucleic acid sequence, especially where the NNT-1 sequence used is a full length genomic sequence or a fragment thereof. Where the intron is not naturally occurring within the NNT-1 DNA sequence (as for most cDNAs), the intron(s) may be obtained from another source. The position of the intron with respect to the 5' flanking sequence and the NNT-1 coding sequence is important, as the intron must be transcribed to be effective. As such, where the NNT-1 nucleic acid sequence is a cDNA sequence, the preferred position for the intron is 3' to the transcription start site, and 5' to the polyA transcription termination sequence. Preferably for NNT-1 cDNAs, the intron will be located on one side or the other (*i.e.*, 5' or 3') of the NNT-1 coding sequence such that it does not interrupt the this coding sequence. Any intron from any source, including any viral, prokaryotic and eukaryotic (plant or animal) organisms, may be used to practice this invention, provided that it is compatible with the host cell(s) into which it is inserted. Also included herein are synthetic introns. Optionally, more than one intron may be used in the vector.

Where one or more of the elements set forth above are not already present in the vector to be used, they may be individually obtained and ligated into the vector. Methods used for obtaining each of the elements are well known to the skilled artisan and are comparable to the methods set forth above (*i.e.*, synthesis of the DNA, library screening, and the like).

The final vectors used to practice this invention are typically constructed from a starting vector such as a commercially available vector. Such vectors may or may not contain some of the elements to be included in the completed vector. If none of the desired elements are present in the starting vector, each element may be individually ligated into the vector by cutting the vector with the appropriate restriction endonuclease(s) such that the ends of the element to be ligated in and the ends of the vector are compatible for ligation. In some cases, it may be necessary to "blunt" the ends to be ligated together in order to obtain a satisfactory ligation. Blunting is accomplished by first filling in "sticky ends" using Klenow DNA polymerase or T4 DNA polymerase in the presence of all four nucleotides. This procedure is well known in the art and is described for example in Sambrook *et al.*, *supra*.

Alternatively, two or more of the elements to be inserted into the vector may first be ligated together (if they are to be positioned adjacent to each other) and then ligated into the vector.

One other method for constructing the vector is to conduct all ligations of the various elements simultaneously in one reaction mixture. Here, many nonsense or nonfunctional vectors will be generated due to improper ligation or insertion of the elements, however the functional vector may be identified and selected by restriction endonuclease digestion.

Preferred vectors for practicing this invention are those which are compatible with bacterial, insect, and/or mammalian host cells. Such vectors include, *inter alia*, pCRII (Invitrogen Company, San Diego, CA), pBSII (Stratagene Company, LaJolla, CA), and pETL (BlueBacII; Invitrogen).

After the vector has been constructed and an NNT-1 nucleic acid has been inserted into the proper site of the vector, the completed vector may be inserted into a suitable host cell for amplification and/or NNT-1 polypeptide expression.

Host cells may be prokaryotic host cells (such as *E. coli*) or eukaryotic host cells (such as a yeast cell, an insect cell, or a vertebrate cell). The host cell, when cultured under appropriate conditions, can synthesize NNT-1 protein which can subsequently be collected from the culture medium (if the host cell secretes it into the medium) or directly from the host cell producing it (if it is not secreted). After collection, the NNT-1 protein can be purified using methods such as molecular sieve chromatography, affinity chromatography, and the like.

Selection of the host cell will depend in part on whether the NNT-1 protein is to be glycosylated or phosphorylated (in which case eukaryotic host cells are preferred), and the manner in which the host cell is able to "fold" the protein into its native tertiary structure (*e.g.*, proper orientation of disulfide bridges, etc.) such that biologically active protein is prepared by the cell. However, where the host cell does not synthesize biologically active NNT-1, the NNT-1 may be "folded" after synthesis using appropriate chemical conditions as discussed below.

Suitable cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO) or 3T3 cells. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. Other suitable mammalian cell lines, are the monkey COS-1 and COS-7 cell lines, and the CV-1 cell line. Further exemplary mammalian host cells include primate cell lines and rodent cell lines,

including transformed cell lines. Normal diploid cells, cell strains derived from *in vitro* culture of primary tissue, as well as primary explants, are also suitable. Candidate cells may be genotypically deficient in the selection gene, or may contain a dominantly acting selection gene. Other suitable mammalian cell lines include but are not limited to, HeLa, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines.

Similarly useful as host cells suitable for the present invention are bacterial cells. For example, the various strains of *E. coli* (e.g., HB101, DH5α, DH10, and MC1061) are well-known as host cells in the field of biotechnology. Various strains of *B. subtilis*, *Pseudomonas spp.*, other *Bacillus spp.*, *Streptomyces spp.*, and the like may also be employed in this method.

Many strains of yeast cells known to those skilled in the art are also available as host cells for expression of the polypeptides of the present invention. Additionally, where desired, insect cells may be utilized as host cells in the method of the present invention (Miller *et al.*, *Genetic Engineering* 8: 277-298 [1986]).

Insertion (also referred to as "transformation" or "transfection") of the vector into the selected host cell may be accomplished using such methods as calcium chloride, electroporation, microinjection, lipofection or the DEAE-dextran method. The method selected will in part be a function of the type of host cell to be used. These methods and other suitable methods are well known to the skilled artisan, and are set forth, for example, in Sambrook *et al.*, *supra*.

The host cells containing the vector (*i.e.*, transformed or transfected) may be cultured using standard media well known to the skilled artisan. The media will usually contain all nutrients necessary for the growth and survival of the cells. Suitable media for culturing *E. coli* cells are for example, Luria Broth (LB) and/or Terrific Broth (TB). Suitable media for culturing eukaryotic cells are RPMI 1640, MEM, DMEM, all of which may be supplemented with serum and/or growth factors as required by the particular cell line being cultured. A suitable medium for insect cultures is Grace's medium supplemented with yeastolate, lactalbumin hydrolysate, and/or fetal calf serum as necessary.

Typically, an antibiotic or other compound useful for selective growth of the transformed cells only is added as a supplement to the media. The compound to be used will be dictated by the selectable marker element present on the plasmid with which the host cell was transformed. For example, where the selectable marker element is kanamycin resistance, the compound added to the culture medium will be kanamycin.

The amount of NNT-1 polypeptide produced in the host cell can be evaluated using standard methods known in the art. Such methods include, without limitation, Western blot analysis, SDS-polyacrylamide gel electrophoresis, non-denaturing gel electrophoresis, HPLC separation, immunoprecipitation, and/or activity assays such as DNA binding gel shift assays.

If the NNT-1 polypeptide has been designed to be secreted from the host cells, the majority of polypeptide may be found in the cell culture medium. Polypeptides prepared in this way will typically not possess an amino terminal methionine, as it is removed during secretion from the cell. If however, the NNT-1

polypeptide is not secreted from the host cells, it will be present in the cytoplasm (for eukaryotic, gram positive bacteria, and insect host cells) or in the periplasm (for gram negative bacteria host cells) and may have an amino terminal methionine.

For intracellular NNT-1 protein, the host cells are typically first disrupted mechanically or osmotically to release the cytoplasmic contents into a buffered solution. NNT-1 polypeptide can then be isolated from this solution.

Purification of NNT-1 polypeptide from solution can be accomplished using a variety of techniques. If the polypeptide has been synthesized such that it contains a tag such as Hexahistidine (NNT-1/hexaHis) or other small peptide at either its carboxyl or amino terminus, it may essentially be purified in a one-step process by passing the solution through an affinity column where the column matrix has a high affinity for the tag or for the polypeptide directly (*i.e.*, a monoclonal antibody specifically recognizing NNT-1). For example, polyhistidine binds with great affinity and specificity to nickel, thus an affinity column of nickel (such as the Qiagen nickel columns) can be used for purification of NNT-1/polyHis. (See for example, Ausubel *et al.*, eds., *Current Protocols in Molecular Biology*, Section 10.11.8, John Wiley & Sons, New York [1993]).

Where the NNT-1 polypeptide has no tag and no antibodies are available, other well known procedures for purification can be used. Such procedures include, without limitation, ion exchange chromatography, molecular sieve chromatography, HPLC, native gel electrophoresis in combination with gel elution, and preparative isoelectric focusing ("Isoprime" machine/technique, Hoefer Scientific). In some cases, two or more of these techniques may be combined to

achieve increased purity. Preferred methods for purification include polyhistidine tagging and ion exchange chromatography in combination with preparative isoelectric focusing.

If it is anticipated that the NNT-1 polypeptide will be found primarily in the periplasmic space of the bacteria or the cytoplasm of eukaryotic cells, the contents of the periplasm or cytoplasm, including inclusion bodies (*e.g.*, gram-negative bacteria) if the processed polypeptide has formed such complexes, can be extracted from the host cell using any standard technique known to the skilled artisan. For example, the host cells can be lysed to release the contents of the periplasm by French press, homogenization, and/or sonication. The homogenate can then be centrifuged.

If the NNT-1 polypeptide has formed inclusion bodies in the periplasm, the inclusion bodies can often bind to the inner and/or outer cellular membranes and thus will be found primarily in the pellet material after centrifugation. The pellet material can then be treated with a chaotropic agent such as guanidine or urea to release, break apart, and solubilize the inclusion bodies. The NNT-1 polypeptide in its now soluble form can then be analyzed using gel electrophoresis, immunoprecipitation or the like. If it is desired to isolate the NNT-1 polypeptide, isolation may be accomplished using standard methods such as those set forth below and in Marston *et al.* (*Meth. Enz.*, 182:264-275 [1990]).

If NNT-1 polypeptide inclusion bodies are not formed to a significant degree in the periplasm of the host cell, the NNT-1 polypeptide will be found primarily in the supernatant after centrifugation of the cell homogenate, and the NNT-1 polypeptide can be

isolated from the supernatant using methods such as those set forth below.

In those situations where it is preferable to partially or completely isolate the NNT-1 polypeptide, purification can be accomplished using standard methods well known to the skilled artisan. Such methods include, without limitation, separation by electrophoresis followed by electroelution, various types of chromatography (immunoaffinity, molecular sieve, and/or ion exchange), and/or high pressure liquid chromatography. In some cases, it may be preferable to use more than one of these methods for complete purification.

B. Chemical Synthesis Methods

In addition to preparing and purifying NNT-1 polypeptide using recombinant DNA techniques, the NNT-1 polypeptides, fragments, and/or derivatives thereof may be prepared by chemical synthesis methods (such as solid phase peptide synthesis) using methods known in the art such as those set forth by Merrifield *et al.*, (*J. Am. Chem. Soc.*, 85:2149 [1964]), Houghten *et al.* (*Proc Natl Acad. Sci. USA*, 82:5132 [1985]), and Stewart and Young (Solid Phase Peptide Synthesis, Pierce Chem Co, Rockford, IL [1984]). Such polypeptides may be synthesized with or without a methionine on the amino terminus. Chemically synthesized NNT-1 polypeptides or fragments may be oxidized using methods set forth in these references to form disulfide bridges. The NNT-1 polypeptides or fragments may be employed as biologically active or immunological substitutes for natural, purified NNT-1 polypeptides in therapeutic and immunological processes.

IV. Chemically Modified NNT-1 Derivatives

Chemically modified NNT-1 compositions (*i.e.*, "derivatives") where the NNT-1 polypeptide is linked to a polymer ("NNT-1-polymers") are included within the scope of the present invention. The polymer selected is typically water soluble so that the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. The polymer selected is usually modified to have a single reactive group, such as an active ester for acylation or an aldehyde for alkylation, so that the degree of polymerization may be controlled as provided for in the present methods. The polymer may be of any molecular weight, and may be branched or unbranched. Included within the scope of NNT-1-polymers is a mixture of polymers. Preferably, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable.

The water soluble polymer or mixture thereof may be selected from the group consisting of, for example, polyethylene glycol (PEG), monomethoxy-polyethylene glycol, dextran, cellulose, or other carbohydrate based polymers, poly-(N-vinyl pyrrolidone) polyethylene glycol, propylene glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, polyoxyethylated polyols (e.g., glycerol) and polyvinyl alcohol.

For the acylation reactions, the polymer(s) selected should have a single reactive ester group. For reductive alkylation, the polymer(s) selected should have a single reactive aldehyde group. A preferred reactive aldehyde is polyethylene glycol propionaldehyde, which is water stable, or mono C1-C10 alkoxy or aryloxy derivatives thereof (see U.S. Patent 5,252,714).

Pegylation of NNT-1 may be carried out by any of the pegylation reactions known in the art, as described for example in the following references: *Focus on Growth Factors* 3: 4-10 (1992); EP 0 154 316; and EP 0 401 384. Preferably, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule (or an analogous reactive water-soluble polymer) as described below.

Pegylation by acylation generally involves reacting an active ester derivative of polyethylene glycol (PEG) with an NNT-1 protein. Any known or subsequently discovered reactive PEG molecule may be used to carry out the pegylation of NNT-1. A preferred activated PEG ester is PEG esterified to N-hydroxysuccinimide ("NHS"). As used herein, "acylation" is contemplated to include without limitation the following types of linkages between NNT-1 and a water soluble polymer such as PEG: amide, carbamate, urethane, and the like, as described in *Bioconjugate Chem.* 5: 133-140 (1994). Reaction conditions may be selected from any of those known in the pegylation art or those subsequently developed, provided that conditions such as temperature, solvent, and pH that would inactivate the NNT-1 species to be modified are avoided.

Pegylation by acylation usually results in a poly-pegylated NNT-1 product, wherein the lysine ε-amino groups are pegylated via an acyl linking group. Preferably, the connecting linkage will be an amide. Also preferably, the resulting product will be at least about 95 percent mono, di- or tri- pegylated. However, some species with higher degrees of pegylation (up to the maximum number of lysine ε-amino acid groups of NNT-1 plus one α-amino group at the amino terminus of

NNT-1) will normally be formed in amounts depending on the specific reaction conditions used. If desired, more purified pegylated species may be separated from the mixture, particularly unreacted species, by standard purification techniques, including, among others, dialysis, salting-out, ultrafiltration, ion-exchange chromatography, gel filtration chromatography and electrophoresis.

Pegylation by alkylation generally involves reacting a terminal aldehyde derivative of PEG with a protein such as NNT-1 in the presence of a reducing agent. Regardless of the degree of pegylation, the PEG groups are preferably attached to the protein via a $-CH_2-NH-$ group. With particular reference to the $-CH_2-$ group, this type of linkage is referred to herein as an "alkyl" linkage.

Derivatization via reductive alkylation to produce a monopegylated product exploits the differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in NNT-1. Typically, the reaction is performed at a pH (see below) which allows one to take advantage of the pK_a differences between the ε-amino groups of the lysine residues and that of the α-amino group of the N-terminal residue of the protein. By such selective derivatization, attachment of a water soluble polymer that contains a reactive group such as an aldehyde, to a protein is controlled: the conjugation with the polymer occurs predominantly at the N-terminus of the protein without significant modification of other reactive groups such as the lysine side chain amino groups. The present invention provides for a substantially homogeneous preparation of NNT-1-monopolymer protein conjugate molecules (meaning NNT-1 protein to which a polymer molecule has been

attached substantially only (*i.e.*, at least about 95%) in a single location on the NNT-1 protein. More specifically, if polyethylene glycol is used, the present invention also provides for pegylated NNT-1 protein lacking possibly antigenic linking groups, and having the polyethylene glycol molecule directly coupled to the NNT-1 protein.

A particularly preferred water-soluble polymer for use herein is polyethylene glycol, abbreviated PEG. As used herein, polyethylene glycol is meant to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono-(C1-C10) alkoxy- or aryloxy-polyethylene glycol.

In general, chemical derivatization may be performed under any suitable conditions used to react a biologically active substance with an activated polymer molecule. Methods for preparing pegylated NNT-1 will generally comprise the steps of (a) reacting an NNT-1 polypeptide with polyethylene glycol (such as a reactive ester or aldehyde derivative of PEG) under conditions whereby NNT-1 becomes attached to one or more PEG groups, and (b) obtaining the reaction product(s). In general, the optimal reaction conditions for the acylation reactions will be determined based on known parameters and the desired result. For example, the larger the ratio of PEG: protein, the greater the percentage of poly-pegylated product.

Reductive alkylation to produce a substantially homogeneous population of mono-polymer/NNT-1 protein conjugate molecule will generally comprise the steps of: (a) reacting an NNT-1 protein with a reactive PEG molecule under reductive alkylation conditions, at a pH suitable to permit selective modification of the α-amino group at the amino

terminus of said NNT-1 protein; and (b) obtaining the reaction product(s).

For a substantially homogeneous population of mono-polymer/NNT-1 protein conjugate molecules, the reductive alkylation reaction conditions are those which permit the selective attachment of the water soluble polymer moiety to the N-terminus of NNT-1. Such reaction conditions generally provide for pK_a differences between the lysine amino groups and the α-amino group at the N-terminus (the pK_a being the pH at which 50% of the amino groups are protonated and 50% are not). The pH also affects the ratio of polymer to protein to be used. In general, if the pH is lower, a larger excess of polymer to protein will be desired (i.e., the less reactive the N-terminal α-amino group, the more polymer needed to achieve optimal conditions). If the pH is higher, the polymer:protein ratio need not be as large (i.e., more reactive groups are available, so fewer polymer molecules are needed). For purposes of the present invention, the pH will generally fall within the range of 3-5, preferably 4-5.

Another important consideration is the molecular weight of the polymer. In general, the higher the molecular weight of the polymer, the fewer number of polymer molecules which may be attached to the protein. Similarly, branching of the polymer should be taken into account when optimizing these parameters. Generally, the higher the molecular weight (or the more branches) the higher the polymer:protein ratio. In general, for the pegylation reactions contemplated herein, the preferred average molecular weight is about 2kDa to about 100kDa (the term "about" indicating ± 1kDa). The preferred average molecular weight is about 5kDa to about 50kDa, particularly preferably about 12kDa to about 25kDa. The ratio of

water-soluble polymer to NNT-1 protein will generally range from 1:1 to 100:1, preferably (for polypegylation) 1:1 to 20:1 and (for monopegylation) 1:1 to 5:1.

Using the conditions indicated above, reductive alkylation will provide for selective attachment of the polymer to any NNT-1 protein having an α-amino group at the amino terminus, and provide for a substantially homogenous preparation of monopolymer/NNT-1 protein conjugate. The term "monopolymer/NNT-1 protein conjugate" is used here to mean a composition comprised of a single polymer molecule attached to an NNT-1 protein molecule. The monopolymer/NNT-1 protein conjugate preferably will have a polymer molecule located at the N-terminus, but not on lysine amino side groups. The preparation will preferably be greater than 90% monopolymer/NNT-1 protein conjugate, and more preferably greater than 95% monopolymer NNT-1 protein conjugate, with the remainder of observable molecules being unreacted (i.e., protein lacking the polymer moiety). The examples below provide for a preparation which is at least about 90% monopolymer/ protein conjugate, and about 10% unreacted protein. The monopolymer/protein conjugate has biological activity.

For the present reductive alkylation, the reducing agent should be stable in aqueous solution and preferably be able to reduce only the Schiff base formed in the initial process of reductive alkylation. Preferred reducing agents may be selected from the group consisting of sodium borohydride, sodium cyanoborohydride, dimethylamine borane, trimethylamine borane and pyridine borane. A particularly preferred reducing agent is sodium cyanoborohydride.

Other reaction parameters, such as solvent, reaction times, temperatures, etc., and means of purification of products, can be determined based on the published information relating to derivatization of proteins with water soluble polymers.

A mixture of polymer-NNT-1 protein conjugate molecules may be prepared by acylation and/or alkylation methods, as described above, and one may select the proportion of monopolymer/ protein conjugate to include in the mixture. Thus, where desired, a mixture of various protein with various numbers of polymer molecules attached (i.e., di-, tri-, tetra-, etc.) may be prepared and combined with the monopolymer/NNT-1 protein conjugate material prepared using the present methods.

Generally, conditions which may be alleviated or modulated by administration of the present polymer/NNT-1 include those described herein for NNT-1 molecules in general. However, the polymer/NNT-1 molecules disclosed herein may have additional activities, enhanced or reduced activities, or other characteristics, as compared to the non-derivatized molecules.

V. Combinations

The NNT-1 polypeptides and fragments thereof, whether or not chemically modified, may be employed alone, or in combination with other pharmaceutical compositions such as, for example, neurotrophic factors, cytokines, interferons, interleukins, growth factors, antibiotics, anti-inflammatories, neurotransmitter receptor agonists or antagonists and/or antibodies, in the treatment of neurological or immunological system disorders.

VI. Antibodies

The NNT-1 polypeptides, fragments, and/or derivatives thereof may be used to prepare antibodies generated by standard methods. Thus, antibodies that react with the NNT-1 polypeptides, as well as reactive fragments of such antibodies, are also contemplated as within the scope of the present invention. The antibodies may be polyclonal, monoclonal, recombinant, chimeric, single-chain and/or bispecific. Typically, the antibody or fragment thereof will be "humanized", *i.e.*, prepared so as to prevent or minimize an immune reaction to the antibody when administered to a patient. The antibody fragment may be any fragment that is reactive with the NNT-1 of the present invention, such as, F_{ab}, $F_{ab'}$, etc. Also provided by this invention are the hybridomas generated by presenting NNT-1 or a fragment thereof as an antigen to a selected mammal, followed by fusing cells (*e.g.*, spleen cells) of the mammal with certain cancer cells to create immortalized cell lines by known techniques. The methods employed to generate such cell lines and antibodies directed against all or portions of a human NNT-1 polypeptide of the present invention are also encompassed by this invention.

The antibodies may be used therapeutically, such as to inhibit binding of NNT-1 to its receptor. The antibodies may further be used for *in vivo* and *in vitro* diagnostic purposes, such as in labeled form to detect the presence of the NNT-1 in a body fluid.

VII. Therapeutic Compositions and Administration Thereof

As used herein, the terms "effective amount" and "therapeutically effective amount" refer to the

amount of NNT-1 necessary to support one or more biological activities of NNT-1 as set forth above.

Therapeutic compositions for treating various neurological disorders or diseases are within the scope of the present invention. Such compositions may comprise a therapeutically effective amount of an NNT-1 polypeptide or fragment thereof (either of which may be chemically modified) in admixture with a pharmaceutically acceptable carrier. The carrier material may be water for injection, preferably supplemented with other materials common in solutions for administration to mammals. Typically, an NNT-1 therapeutic compound will be administered in the form of a composition comprising purified NNT-1 polypeptide or fragment (which may be chemically modified) in conjunction with one or more physiologically acceptable carriers, excipients, or diluents. Neutral buffered saline or saline mixed with serum albumin are exemplary appropriate carriers. Preferably, the product is formulated as a lyophilizate using appropriate excipients (*e.g.*, sucrose). Other standard carriers, diluents, and excipients may be included as desired. An exemplary composition comprises citrate buffer of about pH 4.0-4.5, which may further include NaCl.

The NNT-1 compositions can be systemically administered parenterally. Alternatively, the compositions may be administered intravenously or subcutaneously. When systemically administered, the therapeutic compositions for use in this invention may be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such pharmaceutically acceptable protein solutions, with due regard to pH, isotonicity, stability and the like, is within the skill of the art.

Therapeutic formulations of NNT-1 compositions useful for practicing the present

invention may be prepared for storage by mixing the selected composition having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (*Remington's Pharmaceutical Sciences*, 18th edition, A.R. Gennaro, ed., Mack Publishing Company [1990]) in the form of a lyophilized cake or an aqueous solution. Acceptable carriers, excipients or stabilizers are nontoxic to recipients and are preferably inert at the dosages and concentrations employed, and include buffers such as phosphate, citrate, or other organic acids; antioxidants such as ascorbic acid; low molecular weight polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or polyethylene glycol (PEG).

The NNT-1 composition to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes. Where the NNT-1 composition is lyophilized, sterilization using these methods may be conducted either prior to, or following, lyophilization and reconstitution. The composition for parenteral administration ordinarily will be stored in lyophilized form or in solution.

Therapeutic compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The route of administration of the composition is in accord with known methods, *e.g.* oral, injection or infusion by intravenous, intraperitoneal, intracerebral (intraparenchymal), intracerebroventricular, intramuscular, intraocular, intraarterial, or intralesional routes, or by sustained release systems or implantation device which may optionally involve the use of a catheter. Where desired, the compositions may be administered continuously by infusion, bolus injection or by implantation device. Alternatively or additionally, NNT-1 may be administered locally via implantation into the affected area of a membrane, sponge, or other appropriate material on to which NNT-1 polypeptide has been absorbed.

Where an implantation device is used, the device may be implanted into any suitable tissue or organ, such as, for example, into a cerebral ventricle or into brain parenchyma, and delivery of NNT-1 may be directly through the device via bolus or continuous administration, or via a catheter using continuous infusion.

NNT-1 polypeptide may be administered in a sustained release formulation or preparation. Suitable examples of sustained-release preparations include semipermeable polymer matrices in the form of shaped articles, *e.g.* films, or microcapsules. Sustained release matrices include polyesters, hydrogels, polylactides (U.S. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamine (Sidman *et al*, *Biopolymers*, 22: 547-556 [1983]), poly (2-hydroxyethyl-methacrylate) (Langer *et al.*, *J. Biomed. Mater. Res.*, 15: 167-277 [1981] and Langer, *Chem. Tech.*, 12: 98-105 [1982]), ethylene vinyl acetate (Langer *et al.*, *supra*) or poly-D(-)-3-hydroxybutyric acid (EP 133,988). Sustained-release compositions also

may include liposomes, which can be prepared by any of several methods known in the art (*e.g.*, DE 3,218,121; Epstein *et al.*, *Proc. Natl. Acad. Sci. USA*, 82: 3688-3692 [1985]; Hwang *et al.*, *Proc. Natl. Acad. Sci. USA*, 77: 4030-4034 [1980]; EP 52,322; EP 36,676; EP 88,046; EP 143,949).

In some cases, it may be desirable to use NNT-1 compositions in an *ex vivo* manner, *i.e.*, to treat cells or tissues that have been removed from the patient and are then subsequently implanted back into the patient.

In other cases, NNT-1 may be delivered through implanting into patients certain cells that have been genetically engineered to express and secrete NNT-1 polypeptide. Such cells may be animal or human cells, and may be derived from the patient's own tissue or from another source, either human or non-human. Optionally, the cells may be immortalized. The cells may be implanted into the brain, adrenal gland or into other suitable body tissues or organs of the patient.

In certain situations, it may be desirable to use gene therapy methods for administration of NNT-1 to patients suffering from certain neurological or immunological disorders. In these situations, genomic DNA, cDNA, and/or synthetic DNA encoding NNT-1 or a fragment or variant thereof may be operably linked to a constitutive or inducible promoter that is active in the tissue into which the composition will be injected. This NNT-1 DNA construct, either inserted into a vector, or alone without a vector, can be injected directly into brain or other tissue, either neuronal or non-neuronal.

Alternatively, an NNT-1 DNA construct may be directly injected into muscle tissue where it can be taken up into the cells and expressed in the cells, provided that the NNT-1 DNA is operably linked to a

promoter that is active in muscle tissue such as cytomegalovirus (CMV) promoter, Rous sarcoma virus (RSV) promoter, or muscle creatine kinase promoter. Typically, the DNA construct may include (in addition to the NNT-1 DNA and a promoter), vector sequence obtained from vectors such as adenovirus vector, adeno-associated virus vector, a retroviral vector, and/or a herpes virus vector. The vector/DNA construct may be admixed with a pharmaceutically acceptable carrier(s) for injection.

An effective amount of the NNT-1 composition(s) to be employed therapeutically will depend, for example, upon the therapeutic objectives such as the indication for which NNT-1 is being used, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage may range from about 0.1 μg/kg to up to 10 mg/kg or more, depending on the factors mentioned above. Typically, a clinician will administer the NNT-1 composition until a dosage is reached that achieves the desired effect. The NNT-1 composition may therefore be administered as a single dose, or as two or more doses (which may or may not contain the same amount of NNT-1) over time, or as a continuous infusion via implantation device or catheter.

As further studies are conducted, information will emerge regarding appropriate dosage levels for treatment of various conditions in various patients, and the ordinary skilled worker, considering the therapeutic context, the type of disorder under treatment, the age and general health of the recipient, will be able to ascertain proper dosing.

VIII. Conditions to be Treated with NNT-1

The NNT-1 proteins, fragments and/or derivatives thereof may be utilized to treat diseases and disorders of the central or peripheral nervous system which may be associated with alterations in the pattern of NNT-1 expression or which may benefit from exposure to NNT-1 or anti-NNT-1 antibodies.

NNT-1 protein and/or fragments or derivatives thereof, may be used to treat patients in whom various cells of the central, autonomic, or peripheral nervous system have degenerated and/or have been damaged by congenital disease, trauma, mechanical damage, surgery, stroke, ischemia, infection, metabolic disease, nutritional deficiency, malignancy, and/or toxic agents. More specifically, NNT-1 protein levels may be modulated (up or down regulated) for such indications as Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, Charcot-Marie-Tooth syndrome, Huntington's disease, peripheral neuropathy induced by diabetes or other metabolic disorder, and/or dystrophies or degeneration of the neural retina such as retinitis pigmentosa, drug-induced retinopathies, stationary forms of night blindness, progressive cone-rod degeneration, and the like. Since NNT-1 is also expressed in immune system cells (see Example V below), it may also be useful to treat diseases caused by immune disorders. Further, since NNT-1 is also expressed in hematopoietic cells (see Example V below), it may also be useful to treat diseases caused by disorders of the hematopoietic system.

In addition the NNT-1 proteins, fragments and/or derivatives thereof may be utilized to treat diseases and disorders of the immunological system involving B-cells and/or T cells, preferably B-cells. As shown in Examples IX-XI herein, NNT-1 has an

activity of stimulating B-cell and, to a lesser degree, T-cell production.

There are several primary humoral immunodeficiencies that are potential targets for this factor. Although somewhat rare, these diseases are all chronic and would require long-term treatment. The first is common variable immunodeficiency or CVID which is characterized by somewhat normal levels of circulating B-cells but which lack the capacity to differentiate properly into immunoglobulin producing cells. Individuals with CVID are susceptible to recurrent bacterial infections.

Another NNT-1 target disease is selective IgA deficiency which also results in recurring infections, usually limited to lung, gastrointestinal and urogenital tracts. Selective IgA deficiency is one of the more common of these diseases having a prevalence between 0.03%-0.97% of the population.

Other NNT-1 target diseases include various forms of hypogammaglobulinemia, X-linked aggammaglobulinemia and/or conditions related to one of these diseases such as recurring infections, renal deficiencies, or giardiasis. See, *Clin. Immunol. and Immunopath.*, 40(1):13-24 (1986).

Boosting the humoral immune response to certain vaccines may be another use for NNT-1 polypeptides. For example, antibody production following the administration of oral vaccines is often poor and therefore protects for a limited period of time. The use is envisaged of of NNT-1 as an adjuvant to improve antibody production upon vaccination.

Because of its ability in inhibiting LPS-induced TNF-α production, NNT-1 may find use in the treatment of sepsis. Although many biological response modifier-based approaches to the solution of this very

important clinical problem have not proved to be of any convincing validity, the possibility remains that NNT-1 may succeed there where other therapeutic candidates have failed. The Jarish-Schwarzmann reaction is a clinical condition that bears resemblances to sepsis and is strictly a consequence of TNF toxic action. The use of an anti-TNF antibody has proved to be a clinically successful approach to the treatment of this condition. This is a condition where NNT-1 may exhibit clinical value in terms of its anti-TNF and anti-inflammatory properties.

IX. Assays to Screen for Inhibitors of NNT-1

In some situations, it may be desirable to inhibit or significantly decrease the level of NNT-1 activity. Compounds that inhibit NNT-1 activity could be administered either in an *ex vivo* manner, or in an *in vivo* manner by local or iv injection, or by oral delivery, implantation device, or the like. The assays described below provide examples of methods useful for identifying compounds that could inhibit NNT-1 activity.

For ease of reading, the following definition is used herein for describing the assays:

"Test molecule(s)" refers to the molecule(s) that is under evaluation as an inhibitor of NNT-1, typically by virtue of its potential ability to block the interaction of NNT-1 with its receptor.

The NNT-1 receptor may be isolated, for example, by expression cloning using labeled (*e.g.*, iodinated) NNT-1.

Several types of *in vitro* assays using purified protein may be conducted to identify those compounds that disrupt NNT-1 activity. Such disruption may be accomplished by a compound that typically inhibits the interaction of NNT-1 with its receptor.

In one assay, purified NNT-1 protein or a fragment thereof (prepared for example using methods described above) can be immobilized by attachment to the bottom of the wells of a microtiter plate. Radiolabeled NNT-1 receptor, as well as the test molecule(s) can then be added either one at a time or simultaneously to the wells. After incubation, the wells can be washed and counted using a scintillation counter for radioactivity to determine the degree of NNT-1/receptor binding in the presence of the test molecule. Typically, the molecule will be tested over a range of concentrations, and a series of control "wells" lacking one or more elements of the test assays can be used for accuracy in evaluating the results. A variation of this assay involves attaching the receptor to the wells, and adding radiolabeled NNT-1 along with the test molecule to the wells. After incubation and washing, the wells can be counted for radioactivity.

Several means including radiolabelling are available to "mark" NNT-1. For example, NNT-1 protein can be radiolabelled using 125-I or 35-S. Alternatively, a fusion protein of NNT-1 wherein the DNA encoding NNT-1 is fused to the coding sequence of a peptide such as the *c-myc* epitope. NNT-1-myc fusion protein can readily be detected with commercially available antibodies directed against *myc*.

An alternative to microtiter plate type of binding assays comprises immobilizing either NNT-1 or its receptor on agarose beads, acrylic beads or other types of such inert substrates. The inert substrate containing the NNT-1 or its receptor can be placed in a solution containing the test molecule along with the complementary component (either receptor or NNT-1 protein) which has been radiolabeled or fluorescently labeled; after incubation, the inert substrate can be precipitated by centrifugation, and the amount of

binding between NNT-1 and receptor can be assessed using the methods described above. Alternatively, the insert substrate complex can be immobilized in a column and the test molecule and complementary component passed over the column. Formation of the NNT-1/receptor complex can then be assessed using any of the techniques set forth above, *i.e.*, radiolabeling, antibody binding, or the like.

Another type of *in vitro* assay that is useful for identifying a molecule to inhibit NNT-1 activity is the Biacore assay system (Pharmacia, Piscataway, NJ) using a surface plasmon resonance detector system and following the manufacturer's protocol. This assay essentially involves covalent binding of either NNT-1 or its receptor to a dextran-coated sensor chip which is located in a detector. The test molecule and the complementary component can then be injected into the chamber containing the sensor chip either simultaneously or sequentially, and the amount of binding of NNT-1/receptor can be assessed based on the change in molecular mass which is physically associated with the dextran-coated side of the of the sensor chip; the change in molecular mass can be measured by the detector system.

In some cases, it may be desirable to evaluate two or more test molecules together for use in decreasing or inhibiting NNT-1 activity. In these cases, the assays set forth above can be readily modified by adding such additional test molecule(s) either simultaneously with, or subsequently to, the first test molecule. The remainder of steps in the assay can be as set forth above.

X. Transgenic Mammals

Also included within the scope of the present invention are non-human mammals such as mice, rats,

rabbits, goats, or sheep in which the gene (or genes) encoding the human equivalent of NNT-1 has been disrupted ("knocked out") such that the level of expression of this gene is significantly decreased or completely abolished. Such mammals may be prepared using techniques and methods such as those described in U.S. Patent No. 5,557,032. The present invention further includes non-human mammals such as mice, rats, rabbits, goats, or sheep in which the gene (or genes) encoding the NNT-1 (either the native form of NNT-1 for the mammal or a heterologous NNT-1 gene) is over expressed by the mammal, thereby creating a "transgenic" mammal. Such transgenic mammals may be prepared using well known methods such as those described in U.S. Patent No 5,489,743 and PCT patent application no. WO94/28122, published 8 December 1994.

The following examples are intended for illustration purposes only, and should not be construed as limiting the scope of the invention in any way.

EXAMPLES

Standard methods for library preparation, DNA cloning, and protein expression are set forth in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY [1989]) and in Ausubel *et al*, eds. (*Current Protocols in Molecular Biology*, Wiley, New York, NY [1995]).

Example I: Cloning of cDNA and Genomic Clone for NNT-1

A. Construction of cDNA library

Human T-cell lymphoma cells, Jurkat cells, were grown at 37°C under 5% CO_2 in a RPMI 400 media containing 10% fetal bovine serum. The media was buffered with 10mM HEPES, pH 7.5. After 8 passages the cells were divided into two groups. One group was grown to confluency ($2x10^7$ cells/flask), the RNA harvested from these cells served as the "driver" RNA. The other group was the "tester" group and were activated with the following treatment.

The cells were activated for 8 hours by adding the superantigens Streptococci enterotoxin B and F(TSST) 80 ng/ml; the PKC activator, PMA 50 ng/ml; calcium ionophore A21832 125 ng/ml. The protein translation inhibitor cycloheximide was also added at a concentration of 1 mg/ml. RNA was harvested from the different groups of cells at different time points.

1. Total RNA preparation:

The cells were pelleted by centrifugation at 300 xg for 5 min and washed with PBS (phosphate buffered saline), and resuspended in Ultraspec II (Biotex, Inc., TX), at a concentration of $5x10^6$ cells/ml of Ultraspec II. The cells were then lysed by four passages through a 21-gauge syringe. The homogenate was incubated on ice for 15 min, 0.2 volumes of chloroform was then added, mixed well, and reincubated on ice for a further 10 min, centrifuged at 12000 xg for 30 min in 30 ml corex tubes. Post-centrifugation and supernatant was saved and the residue discarded. 0.05 volumes of the RNA binding resin sold by Biotex as part of the isolation kit was added after the addition of 0.5 volumes of isopropanol.

After pelleting the resin by centrifugation (300 xg for 5 min), the resin was washed twice with 75% RNase-free ethanol, and air dried at 50°C for 10 min. Total RNA was then eluted from the resin by resuspending the resin in 1 volume RNase-free water, vortexing vigorously for 1 min, then centrifugated at 13000 xg for 1 min. The total RNA was then transferred to a new Eppendorf tube and the resin pellet discarded.

2. Poly(A)$^+$ RNA isolation:

Qiagen's Oligotex mRNA isolation system was used as described by the manufacturer; the procedure was repeated twice to obtain pure poly(A)$^+$ RNA. This is especially important for a random primed library to minimize the number of copies of ribosomal RNA in the cDNA. The mRNA integrity was then determined by both spectroscopy and formamide denaturing gel electrophoresis.

The first strand cDNA was synthesized by following the BRL cDNA synthesis protocol. To remove residual mRNA from the target cDNA, the first-strand cDNA reaction was phenol/chloroform extracted and precipitated with 2 M ammonium acetate and 3 volumes of ethanol. The cDNA/mRNA hybrids were then resuspended in 0.3 M NaOH in the presence of 2 mM EDTA and incubated at 68°C for 15 min. The hydrolysis reaction was neutralized with about 1.5 M excess of pure Tris HCl. The cDNA was then phenol/chloroform extracted and reprecipitated with 2 M ammonium acetate and 3 volumes of ethanol, rinsed with 75% ethanol, and resuspended in 7 ml of sterile water. The single strand cDNA was tailed by following the protocol of Boehringer Mannheim tailing kit.

3. Driver mRNA preparation and photobiotinylation:

Poly(A) RNA was isolated as described above. Approximately 20 mg was then photobiotinylated twice with 20 mg photobiotin acetate (Sigma), and reconstituted at a concentration of 1 mg/ml in RNase-free water. Excess photobiotin was removed with water saturated isobutanol, and ethanol precipitated and resuspended in 30 ml DEPC-treated water.

4. Subtractive hybridization reaction:

The photobiotinylated driver mRNA was coprecipitated with the tester cDNA and resuspended in 2 ml RNase-free water. To allow the nucleic acids to go into solution, the preparation was left at room temperature for a few hours with intermittent gentle stirring followed by another 20 hours incubation at 68°C. Photobiotinylated driver was dissolved to a final concentration of 2mg/ml. In general, a concentration of driver RNA of at least 1mg/ml should be used.

5. Post-hybridization hybrid removal:

After the hybridization, streptavidin was added to a final concentration of 0.2 mg/ml and incubated at room temperature for 10 min. The streptavidin was then removed with a phenol/chloroform extraction. After the extraction, the cDNA was precipitated with ethanol.

A pair of primers: AGCGCTACGGTCGACCCG GCG TTT TTT TTT TTT TTT TTT TTT (ACG)X (SEQ ID NO:15) (Sal I T21 anchored primer) and GGA AGG AAA AAA GCG GCC GCT ACA (SEQ ID NO:16)(Not I -N9 primer) were used in PCR to amplify cDNA. The expend PCR kit was used. Fifteen cycles were used to generate enough material for gel fractionation approach to allow for an equal size

representation in the library. To allow for the annealing of the first primer, the annealing temperature of the initial five cycles of the PCR were performed at 35°C for 1 min. The cDNA representing different size fractions were fractionated on a gel. *Sal*I adapters were added to the duplex cDNA, which was then digested with *Not*I and cloned into pSport vector.

B. Isolation of cDNA Clone

The library was screened by expressed sequence tag (est) analysis. Individual clones from this library were randomly picked and sequenced on an Applied Biosystems 373A automated DNA sequencer using vector primer and Taq dye-terminator reactions (Applied Biosystems). The resulting nucleotide sequence obtained from the randomly picked clone NNT-1 was translated, then compared to the existing database of known protein sequences using a modified version of the FASTA program.

One clone (khj1-00008-f2) has about 21% homology at translated amino acid sequence level with CNTF. The entire insert of the cDNA clone was sequenced and found to encode a full-length clone, i.e., it contains Met at the 5′ end and one stop codon upstream of Met and another stop codon at the 3′ end.

The sequence of this full-length cDNA is shown in Figure 1. The predicted amino acid sequence of the protein is shown in Figure 3. The putative signal peptide spanned from amino acid -27 (Met) to amino acid -1 (Ala).

C. Isolation of the Genomic Clone

The genomic DNA of NNT-1 was obtained from a human genomic P1 library (Genome Systems Inc., St. Louis, MO; catalog no. P1-2535). The library was screened using the NNT-1 cDNA as a probe. The cDNA was

radiolabeled using the Amersham Rediprime kit (Amersham, Arlington Heights, IL; catalog no. RPN-1633) and the hybridization and prehybridization solution was: 50 percent formamide, 5 X SSC, 5 X Denhardt's, 0.05 percent sodium pyrophosphate, 0.1 percent SDS, and 100 mg/ml salmon sperm DNA. Prehybridization was for about 1 hour, and hybridization was for about 16 hours at 42°C.

After hybridization, the filters were washed in 0.2 X SSC and 0.1 percent SDS at 42°C for about 30 minutes, and then exposed to film. Two positive clones were identified, and the plasmids containing these clones were purified according to Genome Systems Inc. protocols. The plasmid DNA was then sequenced directly.

The genomic sequence encoding NNT-1 is shown in Figure 2 (SEQ ID NO:3). The gene consists of 3 exons and 2 introns. The coding regions are presented in uppercase, while the noncoding regions, including 5' untranslated region, introns and 3' untranslated region are presented in lower case.

Example II: Preparation of Recombinant Mammalian Protein of NNT-1

An expression vector containing human NNT-1 cDNA and flag-tag peptide was constructed by PCR amplification of the fusion gene. A sense primer with Hind III site at the 5' end:

(5'-AGCAAGCTTCACCATGGACCTCCGAGCAGGGGACTC-3')
(SEQ ID NO: 6)

which encodes amino acid -27 (Met) to amino acid -21 (Asp) and an anti-sense primer with *Not*I site at the 5'

end which encode for flag-tag peptide and the last 8 amino acids of the 3'end

(5'AGCGGGGCCGCACTACTTGRCATCGTCGRCGTCCTTGTACTCGAAGCCATGA GCCCCCAGGTGCAG-3') (SEQ ID NO: 7)

were used in PCR to amplify a fusion gene. The fusion gene was ligated into the P CEP4 vector (Invitrogen Inc., San Diego, CA). The expression vector was transfected into EBNA-1 293 cells with lipofectin (BRL, Gaithersburg, MD) using the manufacturer's recommended method. Forty-eight hours after transfection, both 293 cells and the conditioned medium were harvested and analyzed in Western blot by using the anti-flag-tag antibody (Eastman Kodak Co., New Haven CT). The majority of recombinant protein was found in the 293 cell lysate. Therefore, anti-flag antibody gel (Eastman Kodak Co., New Haven, CT) was used to purify the protein from the 293 cell lysate. A 28-30 kd protein was purified following the manufacturer's protocol. This recombinant protein was used in the biological function analysis (for motor neuron and sympathetic neuron survival assay). The N-terminal amino acid of the protein was determined to be Leu (amino acid 1) indicating that the potential signal peptide was cleaved (amino acid -27 to amino acid -1).

Example III: Preparation of Recombinant E coli NNT-1 Protein

A cDNA clone of NNT-1 encoding amino acids Leu (1) to Phe (198) of SEQ ID NO: 2 was inserted into the vector pAMG21 which is a derivative of pCFM 1656 (ATCC accession number 69576) and contains appropriate restriction sites for insertion of genes downstream from the lux PR promoter (see US Patent No. 5,169,318

for a description of the lux expression system). The host cell used was *E. coli* K12, strain CGSC 6159 (Yale University genetic stock, New Haven, CT). The host cells were transformed with the vector using standard transformation procedures, and were then incubated in 2 XYT medium containing about 50 ul/ml kanamycin at 30°C. Induction of NNT-1 gene product was commenced by adding the autoinducer N-(3-oxohexanoyl)-DL-homoserine lactone to the culture medium to a final concentration of about 30 ng/ml, and the cultures were incubated at either 30°C or 37°C for about 6 hours after which time the cells were examined by microscopy for inclusion bodies.

The majority of NNT-1 protein was found to be located in the inclusion bodies. Therefore, a cell paste was prepared by pelleting the cells. The inclusion bodies were solubilized at low pH and the protein was purified by sequential precipitation. The protein was dialyzed before loading a sample on to SDS-PAGE to assess purity. Coomassie staining of the gel indicated that the protein was at least 95 percent pure.

Example IV: Neurobiological function of NNT-1

A. Chick Motor Neuron Assay

Motor neurons (MN) enriched culture from lumbar spinal cord were prepared from embryonic day E5.5 chicks. MN neurons were enriched by using a 6.8% metrizamide gradient. In brief, lumbar spinal cords were dissected, freed of meninges and DRG. Spinal cords were incubated in papain containing L15 medium (Gibco/BRL, Grand Island, NY) for 20 minutes at 37°C (Worthington Biochemical Corp, Freehold, NJ). Enzymatically softened spinal cord fragments were dissociated into single cells by pipetting. The cell suspension was then layered onto a 6.8% metrizamide

(Serva, Feinbiochemicala, Germany) cushion, and the tube was centrifuged at 500 g for 20 minutes. The interface between metrizamide cushion and cell suspension was collected and diluted into culture medium. The fraction was then gently layered onto a 4% BSA cushion and centrifuged at 280 g for 10 minutes. The pellet was resuspended in culture medium containing L15 medium with 10% fetal bovine serum supplemented with 3.6 mg/ml glucose, 5 ng/ml sodium selenite, 6.25 ng/ml progesterone, 0.1 mg/ml conalbumin, 16 mg/ml putrescine, and 5 mg/ml insulin. 10,000 cells/well were seeded into 96 well tissue culture plates. Serial dilutions of the neurotrophic factor (NNT-1 or CNTF) were added to the culture and incubated for 3 days. At day 3, MTT was added into the culture for 4.5 hours. The formazan product was solubilized, and the plates were read at 570 wavelength with a 650 nm subtraction for visible interference. The optical density (OD) reading is proportional to the number of surviving neurons in culture. The absorbance at 570 nm (increasing neuron survival) in triplicate wells is plotted as a function of final concentration of NNT-1 or CNTF.

Results of the analysis are presented in Figure 7. The absorbance at 570nm is expressed as 1000 fold of the actual reading. The results showed that NNT-1 can support chick motor meuron growth. Its maximal activity reaches about 90% that of CNTF.

B. Chick Sympathetic Neuron Assay

Cultures of primary chick embryo sympathetic chain ganglia were prepared. Briefly, sympathetic ganglia were removed from fertile, pathogen-free chicken eggs that had been incubated for 9 days at 37.6°C in a humidified atmosphere. The ganglia were chemically dissociated by exposure first to Hanks'

Balanced Salt Solution without divalent cations, containing 10mM HEPES buffer pH 7.2 for 10 min at 37°C, and then by exposure to a solution of 0.125% bactotrypsin 1:250 (Difco, Detroit, Michigan) in Hanks' Balanced Salt Solution modified as above for 12 min at 37°C. Trypsinization was stopped by addition of fetal calf serum to a final concentration of 10%.

After this treatment, ganglia were transferred to a solution consisting of Dulbecco's high glucose Modified Eagle's Medium with bicarbonate contain 10% fetal calf serum and 10mM HEPES, pH 7.2 and were mechanically dissociated by trituration approximately 14 times through a 20-gauge, 1" double-hubbed stainless steel needle.

The dissociated ganglia were then plated in culture medium (Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum, 4mM glutamine, 60mg/L penicillin-G, 25mM HEPES, pH 7.2) in 100 mm diameter tissue culture dishes (approximately 40 dissociated ganglia per dish) for two to three hours. This preplating was done in order to separate the nonneuronal cells, which adhere to the dish, from the nerve cells, which do not adhere. After preplating, the nonadherent nerve cells were collected by centrifugation, resuspended in culture medium, and plated in 50 ml per well onto half area 96-well microtiter tissue culture plates at a density of 2500 nerve cells per well. The microtiter wells had been previously exposed to a 1 mg/ml solution of poly-L-ornithine in 10mM sodium borate, pH 8.4 overnight at 4°C, washed in sterile purified water ad air-dried.

Final concentrations of neurotrophic factors to which the cells were exposed are as follows: 1) for the CNTF standard, nine-point serial dilution curves ranged from 100 ng/ml to 6 pg/ml; 2) for the NNT-1 protein, nine-point serial dilutions curves ranged from

100 ng/ml to 0.12 pg/ml. Twenty-five ml of a serial dilution of the sample to be assayed for neurotrophic activity was added to each well and the dishes were incubated for 38-46 hours at 37°C in a humidified atmosphere containing 7.5% CO_2. Then 18 ml per well of a 1.5 mg/ml solution of the tetrazolium dye MTT in Dulbecco's high glucose Modified Eagle Medium with bicarbonate contain 10mM HEPES, pH 7.2 was added, and the cultures were placed in the 37°C incubator for 4.5 hours. Then 75 ml of a solution of 50% N,N-dimethyl formamide containing 20% sodium dodecyl sulfate, pH 4.7 was added to dissolve the crystalline formazan product and the plates were incubated in the 37°C incubator for a minimum of 12 hours. The absorbance at 579nm was determined relative to a 650nm reference for each well using an automatic microtiter plate reader. The resulting absorbance is proportional to the number of living cells in each well, defined as those nerve cells capable of reducing the dye.

Results of the analysis are presented in Figure 8. The results demonstrate that NNT-1 supports chick sympathetic neuron growth.

Example V: Northern Blot Analysis of Tissue Distribution

Northern blots of human tissues were purchased from Clontech (Palo Alto, CA). The Northern blots were probed with a human NNT-1 cDNA probe. Two cDNA fragments spanning the 5′ and 3′ coding region of NNT-1 were labeled and used as a probe to analyze the tissue expression of the NNT-1 gene. The result showed that NNT-1 was expressed as a 2.2 kb transcript in the tissues of spleen, lymph node and peripheral blood lymphocytes, bone marrow and fetal liver, kidney, lung, colorectal adenocarcinoma cells SW480, Hela cell S3, lung carcinoma A 549, chronic myelogenous leukemia K-562

cells, Burkitt's lymphoma Raji cells. The tissue distribution of the gene suggests that the gene may be also involved in development of the immune system or of hematopoietic cells.

Example VI: Chromosome localization of the NNT-1 gene

Chromosome localization of the gene was performed by FISH. A 14 kb genomic fragment was biotinylated with dATP using BRL BioNick labeling kit. (15 C 1 hour). The procedure for FISH was performed according to Heng *et al., Proc Nat Acad Sci USA* 89:9509-9513, 1992. The result showed that the gene is located on chromosome 11 q13 which is close to the human CNTF gene locus (chromosome 11 q12).

Example VII: Isolation of mouse cDNA clone

A mouse partial cDNA clone was isolated by PCR amplification from the mouse 11 day-embryo cDNA (Clontech, Palo Alto, CA) using the human specific primer. The full-length cDNA clone was further obtained by 5' RACE and 3' RACE. The mouse cDNA nucleotide sequence and amino acid sequence are shown in Figs. 4 and 5, respectively. The mouse protein shares 96% identity with the human protein, indicating that the protein is highly conserved throughout evolution. Like the human protein, the mouse protein also contains a potential N-linked glycosylation site at amino acid 2 (Asn).

Example VIII: Comparison of NNT-1 with other members of the family

The amino acid sequence of NNT-1 suggests that the protein belongs to the family of CNTF (SEQ ID NO:12),

which includes IL-11 (SEQ ID NO:8), IL-6 (SEQ ID NO:9), cardiotrophin (SEQ ID NO:11), oncostatin (SEQ ID NO:13) and granulocyte colony-stimulating factor (G-CSF) (SEQ ID NO:10). We compared the amino acid sequence of NNT-1 with all of the members of the family by the computer program PILEUP and the results are shown in Fig. 6. As with all the other members of this family, the secondary structure of the NNT-1 protein was predicted to contain four anti-parallel alpha-helices.

Example IX: Phenotype of NNT-1 Transgenic Mice

A. Phenotype of NNT-1 Transgenic Mice

The protein encoded by the NNT-1 gene has some homology to CNTF and *in vitro* activity in bone marrow and nerve cell assays. Studies of mice transplanted with NNT-1 transfected bone marrow demonstrated mild lymphoproliferation in gastrointestine-associated lymphoid tissues, but no other obvious phenotypic changes.

Materials and Methods

Species: Mouse **Strain:** BDF1 **Age:** 17 wks (120 days)

Test article: NNT-1 (WX240) **Sex:** Male/Female

Treatment Groups

GROUP	MOUSE NO.
Negative	22, 23, 45, 63, 65
Positive	35, 36, 46, 60, 62

There were no obvious abnormalities detected in the two groups.

Gross necropsy was performed with selected tissues fixed in buffered zinc formalin for histopathologic examination [brain, heart, kidneys, adrenals, duodenum, pancreas, bladder, liver, lungs, spleen, any gross lesions]. Tissues were fixed overnight before routine histologic processing. The data were analyzed using the *JMP* (SAS Institute, Cary, NC) software program.

Tests: organ weights, body weight, histopathology, immunohistology, Northern blot.

The following treatment-related changes were present in the NNT-1-transgenic mice:

The spleen had moderate to marked reactive lymphoid hyperplasia (FIG. 10) involving the follicular (B cell) and periarteriolar (T cell) areas in the transgenic mice. The lymphoid hyperplasia was most prominent in the high expresser mouse #62 (FIG. 10), and correlated well with the splenomegaly seen at necropsy. The other high expresser mouse #60 had only mild hyperplasia of the lymphoid areas accompanied by massive diffuse extramedullary hematopoiesis of all three lineages. Although it is difficult to make any general conclusions about the splenic effects of NNT-1 on the basis of these two high expresser mice, the lymphoproliferation seen in mouse #62 is in agreement with our findings with the injected protein (See Example X A below), while the EMH found in mouse #60 may reflect an *in vivo* correlate of the previous *in vitro* bone marrow culture findings.

The liver of mouse #60 had multifocal aggregates of lymphocytes and plasma cells infiltrating perivascular spaces and expanding into the adjacent sinusoids in a peculiar pattern that resembled intrahepatic "islands of lymphopoiesis" (FIG. 12). By immunohistochemistry, the lymphoid aggregates were

composed of B220+ cells and CD3+ cells. Similar but milder and typically perivascular lymphoid infiltrates were also found in mouse #62. Other changes found in the liver occurred sporadically in individual mice in the control and/or transgenic groups.

The gastrointestinal tract had minimal to moderate reactive lymphoid hyperplasia of Peyer's patches (gut-associated lymphoid tissue). Similarly, the cervical and mesenteric lymph nodes were more reactive in the transgenic mice than in the controls, although this change was not as prominent a feature of this study than our study with injected NNT-1 protein (See Example X A below).

The bone marrow, central and peripheral nervous systems of the transgenic mice appeared normal. Generally, the changes in the other tissues were sporadically found in one or more animals in the negative control and/or transgenic groups, and were not interpreted to be transgene-related.

The data from this study indicate that the NNT-1 transgenic mice have an interesting phenotype characterized by proliferation of T and B lymphocytes and plasma cells in multiple peripheral tissues, including the spleen, lymph nodes, gut-associated lymphoid tissue, kidneys and liver. NNT-1 may also induce extramedullary hematopoiesis in some peripheral tissues, such as the spleen and pancreas, in the absence of significant changes in the peripheral blood or bone marrow. Thus, the data from the NNT-1 transgenic mice generally support the findings from our 7-day mouse study with injectable NNT-1 protein (Example X A below), which induced proliferation of lymphoid tissues without detectable effects on bone marrow or central nervous system.

Interestingly, the glomerulonephritis detected in the two high expresser NNT-1 transgenic female mice closely resembles the spontaneous glomerulonephritis seen in the MRL/lpr (Fas-deficient) mice, which develop an early-onset SLE-like autoimmune syndrome associated with polyclonal B-cell activation, multiple autoantibodies, circulating immune complexes and accumulation of an unusual population of double negative (CD4- CD8- TCRab+ CD3+) T cells that also express the CD45R isoform called B220+, which is normally a marker of B cells (Singer *et al., Curr. Opin. Immunol.*, 6:913-920, 1994). Moreover, some of the biologic effects of NNT-1 also mimic those of interleukin-6, which (like CNTF, LIF and IL-11) utilizes the gp130 signaling transducer and has pleiotropic effects on the liver, kidney, brain, skin, immune and hematopoietic systems (Ryffel *et al., Int. Rev. Exp. Pathol.*, 34A:79-89, 1993). Therefore, it will be important to determine if the lymphocytes found in the peripheral blood or tissues might have an unusual phenotype with dual expression of T and B cell markers by flow cytometric analysis.

B. FACS Immunophenotyping of NNT-1 Transgenic Founders

Tissues analyzed

Peripheral blood samples were obtained via retro-orbital bleeds. Nine samples from each group of founder littermate control and NNT-1 positive (by PCR) mice were received; none were clotted. Approximately 20-40ul of blood per sample was incubated first with Fc block antibody followed by fluorescent antibodies for various cell surface antigens.

Antibodies were chosen for markers to differentiate most hematopoietic cell populations in

circulating peripheral blood. Also, some B and T-cell activation/differentiation markers were chosen based on origin of library for this expressed sequence tag (est). The library was created from Jurkat cells (a T-cell line) activated with toxic shock syndrome toxin (TSST).

Antibodies

Fc Block (CD32/16) - as part of pre-incubation to block non-specific binding, a total of 21 antibodies were used. Data was analyzed as single color histograms.

Rat IgG fluorescein isothiocyanate (FITC) + Rat IgG phycoerythren (PE)

Ham IgG FITC + Ham IgG PE

CD45 FITC + GR-1 (CD97) PE ----- Pan leukocyte vs granulocyte

CD4 FITC + CD8 PE -------T-cell subsets Helper vs killer

Th1.2 FITC + B220 PE -----T-cell vs pan B-cell marker

CD69 FITC + CD28 PE ----- Activation markers for T & B or just T-cells

CD3 FITC + CTLA4 PE ------Pan T-cell vs T-cell activation

ckit FITC + Sca-1 PE ------ myeloid and progenitor cells vs progenitors and peripheral lymphocytes

CD40 FITC + CD40L PE ----- B-cell diff. Ag vs T-cell ligand for same

CD62L FITC + CD54 PE ----- Activating adhesion molecules on B and T-cells

CD34 FITC (data not analyzed)

Results

A pronounced increase was observed in absolute cell numbers for four of the NNT-1 positive animals for B220+, CD40+, CD62L+, and CD54+ cells.

These four animals (#24,35,60,62) were later confirmed as expressers by Northern blot. The increase in B220+ and CD40+ cells ranged from 2-4 fold above the control. CD62L+(LECAM) and CD54+(ICAM) ranged from 1.5-3 fold above the control group. Markers showing an increase in three of the four expressers included Sca-1 (2-6 times control) and ckit (2-3 times control). Additional markers including CD3, CD4, CD8, Thy1.2 showed modest increases in two of the four expressers, though not in a consistent fashion (although these are all T-cell markers, they were not all positive in the same expressers). GR1 showed an increase in one of the expressers, but there was an even higher GR1+ cell number in one of the control animals, so this is probably not significant. The rest of the antibodies were either not positive, not significantly different, or in the case of CD34, impossible to analyze.

Summary

A very definite increase in the absolute number of circulating lymphoid cells is observed in these mice. This increase in the lymphoid population seems to consist primarily of B-cells, although some increase in T-cell numbers may be seen as well. Neither lymphoid population appears to exhibit an increase in activated cell types. Little to no effect is seen on the circulating myeloid cell population. Increases in ckit and Sca-1 do not necessarily correlate to an increase in progenitor cells as these markers are found on mature circulating cells as well.

The data is suggestive of a B-cell directed proliferation as these cell numbers all correlate well with expression. The increases in some of the animals' T-cells could possibly be a secondary effect of some other factor(s) being produced by the increased B-cells. One interesting observation with regard to the

B-cells is a slight but very consistent difference between the number of B220+ cells and CD40+ cells. Although both of these are B-cell markers, CD40 is also found on dendritic cells as well.

Example X: Lymphoid Hyperplasia in Mice Injected with NNT-1

A. A Seven-Day Exploratory Intravenous/Subcutaneous Study in NNT-1 Treated BDF1 Female Mice

The protein encoded by NNT-1 had some homology to CNTF and *in vitro* activity in bone marrow and nerve cell assays. The objective of this study was to determine the systemic effects and potential toxicity of NNT-1 protein when administered daily to mice for 7 days.

Materials and Methods

Twenty 6-week old, female BDF1 mice were used for the study. The mice were randomly assigned into the following treatment groups (n=5/group):

1. PBS buffer control (intravenous dosing once daily for 7 days)
2. NNT-1 at 1.5 mg/kg (intravenous)
3. NNT-1 at 0.15 mg/kg (intravenous)
4. NNT-1 at 1.5 mg/kg (subcutaneous)

The mice were not fasted prior to gross necropsy. One hour prior to necropsy (24 hrs after last dosing), the mice were given an intraperitoneal injection of BrdU (at 50 mg/kg for cell proliferation studies). Blood was obtained via cardiac puncture for the determination of hematology (hemoglobin, hematocrit, red blood cell count, platelet count, mean platelet volume, total and differential leukocyte counts) and clinical chemistry parameters (alanine aminotransferase, aspartate aminotransferase, alkaline

phosphatase, lactate dehydrogenase, glucose, urea nitrogen, creatinine, total protein, albumin, globulin, calcium, phosphorus, total bilirubin, uric acid, cholesterol and triglycerides).

Gross necropsy was performed with selected tissues fixed in buffered zinc formalin for histopathologic examination [adrenals, bone marrow, bone (femur), brain, cecum, proximal and distal colon, duodenum, esophagus, heart, ileum, jejunum, kidneys, liver, lungs, mammary glands, ovaries, pancreas, skeletal muscle, skin, spleen, stomach, thymus, thyroid glands, trachea, urinary bladder, uterus, vagina, white and brown adipose tissue, any gross lesions]. Tissues were fixed overnight before routine histologic processing. Organ weights were obtained for the spleen, liver, stomach, kidneys and thymus.

Results

Spleen. There was prominent lymphoid hyperplasia in the white pulp of the spleen with enlargement of the periarteriolar lymphoid sheaths (T-cell areas) and follicles (B-cell areas) in the NNT-1-treated groups. However, the extent of extramedullary hematopoiesis was not apparently increased in these groups, which suggests that this protein may have stimulatory or growth factor-like effects on lymphocytes rather than on hematopoietic cells in vivo.

Lymph node. The NNT-1-treated mice had mild to marked reactive lymphoid hyperplasia of the follicular (B-cell) and paracortical (T-cell) areas of the lymph node cortex. Although this change may reflect an early immune response to the recombinant protein, the degree of generalized reactive lymphoid hyperplasia that was present in the spleen, lymph nodes, Peyer's patches and bone marrow suggests that

this may be a specific treatment-related effect of NNT-1.

Summary and Conclusions

The most significant finding derived from this study was that NNT-1-treatment of mice for 7 days appeared to induce proliferation of lymphoid tissues, particularly in the spleen and lymph nodes. However, this protein did not appear to have any detectable effects on the hematopoietic or central nervous systems under the conditions of this study.

B. FACS Analysis of NNT-1 Injected Mice

Reagents and Mice. Recombinant human NNT-1 and rhIL-1 were from Amgen Inc., Thousand Oaks, CA. LPS (*Escherichia coli* 0111:B4) was purchased from LIST Biologic Laboratories, Campbell, CA. Female Balb/c mice of approximately 20 g were purched from Charles River Laboratories, Wilmington, MA. Mice were housed in rooms maintained at constant temperature and humidity and subjected to 12 hour light/dark cycle. Mice received standard laboratory diet and water *ad libitum*. Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with national and international laws and policies (U.S. National Research Council, Guide for the Care and Use of Laboratory Animals, 1996).

Lymph Node Weight and Cell Counts. For seven consecutive days mice received a daily i.p. injection of 5 mg/Kg of NNT-1 or buffer. Twenty-four hours after the seventh injection, mice were sacrificed for the collection of peripheral (cervical and axyllary) lymph nodes. Lymph nodes were pooled, weighed and homogenized so as to prepare a cell suspension. Cells were then counted with a Sismex cell counter (Toa

Medical Corporation, Kobe, Japan), stained by direct IF using a rat anti-mouse anti-CD45R (anti-B220) MAb (Pharmingen, San Diego, CA) and analyzed in a FACSCAN using the Cell Quest software (Becton and Dickinson, San Jose, CA).

Statistical Analysis. Results are expressed as mean±SD. TNF values were log-transformed to lessen their skewed distribution and bring them to normality. The Shapiro-Wilks test was used to analyze the normality of their distribution before and after transformation. Differences between groups were analyzed by the Student's t test. Since BW was repeatedly measured in each individual, differences in BW within and between groups were tested by the analysis of variance (ANOVA) for repeated measures.

Lymph Node Weight and Cell Counts. NNT-1 treatment increased the counts of total and CD45-positive cells in peripheral lymph nodes (FIG. 17).

Example XI: NNT-1 Shows In-Vivo Activities Characteristic of Cytokines of the IL-6 Family

Reagents, mice and statistical analyses are as set forth in Example X B above.

Serum amyloid A (SAA) Induction, Potentiation of Corticosterone and IL-6 Induction by IL-1 and Inhibition of LPS-Induced TNF. NNT-1 was given i.p. at a dose of 5 mg/kg, alone or in association with IL-1 (100 ng/mouse) or LPS (100 ng/mouse). Control mice received the solvent for NNT-1 (10 mM acetate in saline). Blood was taken from the retro-orbital plexus 8 hours after the administration of NNT-1 or saline for SAA determination, 2 hours after for corticosterone and IL-6 and 1.5 hours after for TNF. Experiments were conducted on groups of five or ten mice.

SAA, IL-6 and TNF were measured in serum by ELISA using commercially available kits (Biogen, Camarillo, CA); results were expressed in μg, ng and pg/ml, respectively. Corticosterone was measured by RIA using a commercially available kit (ICB Biomedical, Costa Mesa, CA); results were expressed in ng/ml.

SAA Induction, Potentiation of Corticosterone and IL-6 Induction by IL-1 and Inhibition of LPS-Induced TNF. NNT-1 induced circulating SAA (FIG. 13).

NNT-1 potentiated the induction by a low dose of IL-1 of either serum corticosterone or IL-6 (FIGS. 14 and 15). NNT-1 also showed the ability to increase the circulating levels of corticosterone when it was injected alone.

NNT-1 inhibited the induction by LPS of serum TNF (FIG. 16).

Summary of Results

Inflammatory processes are accompanied by the production of TNF, a cytokine largely responsible for the tissue damage and functional impairement that distinguish inflammation-related pathology. Often IL-1 is co-produced with TNF and is also thought to be a pathogenetic mediator during inflammation. Corticosteroids are broad spectrum and very powerful anti-inflammatory agents which are induced by IL-1 via an efficient negative feed-back circuit. Corticosteroids inhibit both TNF and IL-1 production. IL-6, which is also induced by both TNF and IL-1, is also able to inhibit TNF and IL-1 production via another negative feed-back circuit.

The ability of NNT-1 to induce corticosteroids and IL-6, at least in presence of IL-1,

suggests that this molecule has the ability of potentiating two physiological anti-inflammatory circuits. This may lead to an accelarated inhibition of the production of TNF and IL-1 and to an accelerated resolution therefore of inflammatory processes. In addition to and independently of the induction of corticosteroids and IL-6 production, NNT-1 exhibits the property of directly blocking TNF production. This interestingly adds to the anti-inflammatory features outlined above.

Deposit of DNA

E. coli cells DH10B containing the vector P1 encoding human genomic DNA for NNT-1 (NNT-g-PI) and *E. coli* cells DH10B containing the vector PSPORT encoding human cDNA for NNT-1 have been deposited with the ATCC (American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA) on January 21, 1997 and assigned accession numbers 98294 and 98295, respectively.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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ELLIOTT, GARY S.
SARMIENTO, ULLA
SENALDI, GIORGIO

(ii) TITLE OF INVENTION: THE NEUROTROPHIC FACTOR NNT-1

(iii) NUMBER OF SEQUENCES: 16

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/792,019
(B) FILING DATE: 03-FEB-1997

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(A) NAME: COOK, ROBERT R.
(B) REGISTRATION NUMBER: 31,602
(C) REFERENCE/DOCKET NUMBER: A-442B

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 797 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 90..764

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 171..764

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(A) NAME/KEY: sig_peptide

(B) LOCATION: 90..170

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(A) LENGTH: 225 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 137..138
(D) OTHER INFORMATION: /product= "INTERVENING UNSEQUENCED REGION OF >1KB"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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AGTCTAAACT AATCTGAAAC TTTTAAAGTA ACCCAGTCCT TAAACCTAAC CTAGCCCAAT 360

GCCAATTATA TCTACCCTAG CCAAACCCTA ACTGCCTTTG CCAGTCCAAA GTGTCCACTG 420

AATCCTCACC TTGGTCCTCA CTGAAAATCC CAGAAAAGCA TATTTCCCCA CTGCCCACAT 480

CCCTCCTTAC AGCACCCAAC CCTGGCCTCT GGACTCCTGG TATCCTGGGA TGTCCAAACT 540

CTGCAGTGCC ATCAGCCAAC AAGCCCGACT CGTCAAATGC ACCTCTCTCC CTTCCTGTCC 600

CCACCCTTGC AGGCTGATGG AAAGGCCTCA TTGAAGTCCA ACTTTTCCCC ACCTAACACC 660

AAGAACGGGG TGAACCTCCA CACTGCCACC GTTCCCTGAG AGTGAGCACT AAATCTCCTT 720

CAATCTAACC CCACCCTACA CTTCCCACAC TCAGGAATCA CATCCTAGAA TATACCCAAA 780

ACTAAGCCCC ATAAGGCAGC CCGACCCTAG TGGTCTAACC CTATACCTTG CTTCCTATGG 840

GTGAGTCTGT TCTTGGCGGC CGCCTCTCTC CTGCTTCCTC CCTTAGAGCT GACTGTGCTC 900

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CACAGGGGAC CCAGGGCCTG GCCCCTCCAT CCAGAAAACC TATGACCTCA CCCGCTACCT 1500

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TCCCTGGGCC CCAGCCGCTC CCCCGAGGGT TGGAAAGGGC TCTGCCCTCT TCCCTATACC 2460

ATGCTGTCTT CCATAGCCTT CCTCCTGTCC TACTCATGAG ACTGCCTCCA TTTCTTCCTT 2520

CTGCAACCCT GCTCCTATCA GCTGAACCCT TCTTTCGGAG TGTTAGTGAG TACCCGTCTC 2580

TCCCCAGCCC CTCAGCTGGT GGGCCTGGGT GTGTCAGCGG CAAATGGGGC TCTGGTTCCA 2640

ATGGGCCACT CTCATCTCTC TCTTGTTCCT TGTGCAGAAA ACCTTTGCTT CACTCCACTG 2700

CCCTCTCTAG TTCCCGACCC TTTTTCTCTC CTGGCTTTCC CTGCCAAATT TCTCCAAGGA 2760

GTGGTCTACA CCCTCTGCCT CCACTTCCTC TCCACCCACT CACTTCTTAA CCCCCTGCAA 2820

TCTGGCTTCC AGGCCCCAGC AATGGTTCTC TCCAAGGTCG TCAGGCACCT CCTTGCCAAG 2880

CCCGACAGTG TTTTGAAGGC TCATTCTCCT TGCTGTCTGT TTTGCAGCCA CACTGCTGAG 2940

CGCTGCTGCC TTCTCGAACT CCTCTTCCTT GGTCTCTGCA CTCTCCTGGG CCACCTTCTA 3000

CCTCTCCAGC TCCTCCAGGC TCCTCTTCCT CTCTGTCCTG CCCCCACAGC GGGCACTCTC 3060

CCAAGGTTTG CCCACCCAGC CAATCAGCAC GTCCTTCCTG AGCGTCTTGT GCGTCTCCTC 3120

CTCCTCCTTT TTCTACGCCT CTCCATTGGA GAGCTCACCA CCGCCACTGC TTCAACTGTC 3180

ACCTGCATAC AAATGATATC CTTATTGGAA AAACTCAGGG AGGCCATGAA CAAAGAAGCC 3240

TAGCATGGAG ACAGGGCCAG TGTCAGGGGA CACAAAAAAT AGAAACTTTG GGAGCAGGTA 3300

TCTCCTTGGT GGTGAGCCAG CGGCTCTGCC CTCCTCCTTC CCCATCACCC TCTCCTTTTC 3360

ACAGCTGAAC TACCTGGGCC CCCCTTTCAA CGAGCCAGAC TTCAACCCTC CCCGCCTGGG 3420

GGCAGAGACT CTGCCCAGGG CCACTGTTGA CTTGGAGGTG TGGCGAAGCC TCAATGACAA 3480

ACTGCGGCTG ACCCAGAACT ACGAGGCCTA CAGCCACCTT CTGTGTTACT TGCGTGGCCT 3540

CAACCGTCAG GCTGCCACTG CTGAGCTGCG CCGCAGCCTG GCCCACTTCT GCACCAGCCT 3600

CCAGGGCCTG CTGGGCAGCA TTGCGGGCGT CATGGCAGCT CTGGGCTACC CACTGCCCCA 3660

GCCGCTGCCT GGGACTGAAC CCACTTGGAC TCCTGGCCCT GCCCACAGTG ACTTCCTCCA 3720

GAAGATGGAC GACTTCTGGC TGCTGAAGGA GCTGCAGACC TGGCTGTGGC GCTCGGCCAA 3780

GGACTTCAAC CGGCTCAAGA AGAAGATGCA GCCTCCAGCA GCTGCAGTCA CCCTGCACCT 3840

GGGGGCTCAT GGCTTCTGAC TTCTGACCTT CTCCTCTTCG CTCCCCCTTC AAACCCTGCT 3900

CCCACTTTGT GAGAGCCAGC CCTGTATGCC AACACCTGTT GAGCCAGGAG ACAGAAGCTG 3960

TGAGCCTCTG GCCCTTTCCT GGACCGGCTG GGCGTGTGAT GCGATCAGCC CTGTCTCCTC 4020

CCCACCTCCC AAAGGTCTAC CGAGCTGGGG AGGAGGTACA GTAGGCCCTG TCCTGTCCTG 4080

TTTCTACAGG AAGTCATGCT CGAGGGAGTG TGAAGTGGTT CAGGTTGGTG CAGAGGCGCT 4140

CATGGCCTCC TGCTTCTTGC CTACCACTTG GCCAGTGCCC ACCCAGCCCC TCAGGTGGCA 4200

CATCTGGAGG GCAGGGGTTG AGGGGCCACC ACCACACATG CCTTTCTGGG GTGAAGCCCT 4260

TTGGCTGCCC CACTCTCCTT GGATGGGTGT TGCTCCCTTA TCCCCAAATC ACTCTATACA 4320

TCCAATTCAG GAAACAAACA TGGTGGCAAT TCTACACAAA AAGAGATGAG ATTAACAGTG 4380

CAGGGTTGGG GTCTGCATTG GAGGTGCCCT ATAAACCAGA AGAGAAAATA CTGAAAGCAC 4440

AGGGGCAGGG ACAGACCAGA CCAGACCCAG GAGTCTCCAA AGCACAGAGT GGCAAACAAA 4500

ACCCGAGCTG AGCATCAGGA CCTTGCCTCG AATTGTCTTC CAGTATTACG GTGCCTCTTC 4560

TCTGCCCCCT TTCCCAGGGT ATCTGTGGGT TGCCAGGCTG GGGAGGGCAA CCATAGCCAC 4620

ACCACAGGAT TTCCTGAAAG TTTACAATGC AGTAGCATTT TGGGGTGTAG GGTGGCAGCT 4680

CCCCAAGGCC CTGCCCCCCA GCCCCACCCA CTCATGACTC TAAGTGTGTT GTATTAATAT 4740

TTATTTATTT GGAGATGTTA TTTATTAGAT GATATTTATT GCAGAATTTC TATTCTTGTA 4800

TTAACAAATA AAATGCTTGC CCCAGAACTT AGTCTCTTTG CCCAGCCTCA CCCCTCCTGG 4860

TGCTCATCAG ACTCTTGCCA CCCCTGGCTC CCACTCCCTG CTTGCCTCTG GTGGAGCTGC 4920

ACAGAGCTCT GGGAAGAGGC CCTCTTCCTC CCCGCACTGG GGCGATGGGC GCACCTCAGA 4980

CTTACCCACT GCTGCTGCCA CCACCAACCC CTTGATCCCT CAGTCCTCCC ACACAGCTTC 5040

TGTCCACCCC AGGTTTCCCT CACCCCACCT TTGCTAAGTC TTCCTCA 5087

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 819 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 95..769

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 176..769

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 95..175

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
TATTATTAAA GCTTCGCCGG AGCCGCGGCT CGCCCTCCCA CTCCGCCAGC CTCTGGGAGA      60

GGAGCCGCGC CCGGCCGGCC CGGCCCCCAG CCCC ATG GAC CTC CGA GCA GGG          112
                                      Met Asp Leu Arg Ala Gly
                                      -27     -25

GAC TCG TGG GGG ATG TTA GCT TGC CTA TGC ACG GTG CTG TGG CAC CTC        160
Asp Ser Trp Gly Met Leu Ala Cys Leu Cys Thr Val Leu Trp His Leu
    -20                 -15                 -10

CCT GCA GTG CCA GCT CTT AAT CGC ACA GGA GAT CCA GGC CCT GGC CCC        208
Pro Ala Val Pro Ala Leu Asn Arg Thr Gly Asp Pro Gly Pro Gly Pro
 -5                  1                   5                  10

TCC ATC CAG AAA ACC TAT GAC CTC ACC CGC TAC CTG GAG CAT CAA CTC        256
Ser Ile Gln Lys Thr Tyr Asp Leu Thr Arg Tyr Leu Glu His Gln Leu
            15                  20                  25

CGC AGC TTA GCT GGG ACC TAC CTG AAC TAC CTG GGG CCC CCT TTC AAC        304
Arg Ser Leu Ala Gly Thr Tyr Leu Asn Tyr Leu Gly Pro Pro Phe Asn
        30                  35                  40

GAG CCT GAC TTC AAT CCT CCT CGA CTG GGG GCA GAA ACT CTG CCC AGG        352
Glu Pro Asp Phe Asn Pro Pro Arg Leu Gly Ala Glu Thr Leu Pro Arg
    45                  50                  55

GCC ACG GTC AAC TTG GAA GTG TGG CGA AGC CTC AAT GAC AGG CTG CGG        400
Ala Thr Val Asn Leu Glu Val Trp Arg Ser Leu Asn Asp Arg Leu Arg
60                  65                  70                  75

CTG ACC CAG AAC TAT GAG GCG TAC AGT CAC CTC CTG TGT TAC TTG CGT        448
Leu Thr Gln Asn Tyr Glu Ala Tyr Ser His Leu Leu Cys Tyr Leu Arg
                80                  85                  90
```

```
GGC CTC AAC CGT CAG GCT GCC ACA GCT GAA CTC CGA CGT AGC CTG GCC        496
Gly Leu Asn Arg Gln Ala Ala Thr Ala Glu Leu Arg Arg Ser Leu Ala
            95                  100                 105

CAC TTC TGT ACC AGC CTC CAG GGC CTG CTG GGC AGC ATT GCA GGT GTC        544
His Phe Cys Thr Ser Leu Gln Gly Leu Leu Gly Ser Ile Ala Gly Val
        110                 115                 120

ATG GCG ACG CTT GGC TAC CCA CTG CCC CAG CCT CTG CCA GGG ACT GAG        592
Met Ala Thr Leu Gly Tyr Pro Leu Pro Gln Pro Leu Pro Gly Thr Glu
    125                 130                 135

CCA GCC TGG GCC CCT GGC CCT GCC CAC AGT GAC TTC CTC CAG AAG ATG        640
Pro Ala Trp Ala Pro Gly Pro Ala His Ser Asp Phe Leu Gln Lys Met
140                 145                 150                 155

GAT GAC TTC TGG CTG CTG AAG GAG CTG CAG ACC TGG CTA TGG CGT TCA        688
Asp Asp Phe Trp Leu Leu Lys Glu Leu Gln Thr Trp Leu Trp Arg Ser
                160                 165                 170

GCC AAG GAC TTC AAC CGG CTT AAG AAG AAG ATG CAG CCT CCA GCA GCT        736
Ala Lys Asp Phe Asn Arg Leu Lys Lys Lys Met Gln Pro Pro Ala Ala
            175                 180                 185

TCA GTC ACC CTG CAC TTG GAG GCA CAT GGT TTC TGACCTCTGA CCCTTAACCC      789
Ser Val Thr Leu His Leu Glu Ala His Gly Phe
        190                 195

CCACACCTCC AGGCCCAGTC AGCTGTGCTT                                      819
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 225 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Asp Leu Arg Ala Gly Asp Ser Trp Gly Met Leu Ala Cys Leu Cys
-27     -25                 -20                 -15

Thr Val Leu Trp His Leu Pro Ala Val Pro Ala Leu Asn Arg Thr Gly
    -10                 -5                  1                   5

Asp Pro Gly Pro Gly Pro Ser Ile Gln Lys Thr Tyr Asp Leu Thr Arg
                10                  15                  20

Tyr Leu Glu His Gln Leu Arg Ser Leu Ala Gly Thr Tyr Leu Asn Tyr
            25                  30                  35

Leu Gly Pro Pro Phe Asn Glu Pro Asp Phe Asn Pro Pro Arg Leu Gly
        40                  45                  50

Ala Glu Thr Leu Pro Arg Ala Thr Val Asn Leu Glu Val Trp Arg Ser
    55                  60                  65

Leu Asn Asp Arg Leu Arg Leu Thr Gln Asn Tyr Glu Ala Tyr Ser His
70                  75                  80                  85

Leu Leu Cys Tyr Leu Arg Gly Leu Asn Arg Gln Ala Ala Thr Ala Glu
```

90 95 100

Leu Arg Arg Ser Leu Ala His Phe Cys Thr Ser Leu Gln Gly Leu Leu
105 110 115

Gly Ser Ile Ala Gly Val Met Ala Thr Leu Gly Tyr Pro Leu Pro Gln
120 125 130

Pro Leu Pro Gly Thr Glu Pro Ala Trp Ala Pro Gly Pro Ala His Ser
135 140 145

Asp Phe Leu Gln Lys Met Asp Asp Phe Trp Leu Leu Lys Glu Leu Gln
150 155 160 165

Thr Trp Leu Trp Arg Ser Ala Lys Asp Phe Asn Arg Leu Lys Lys Lys
170 175 180

Met Gln Pro Pro Ala Ala Ser Val Thr Leu His Leu Glu Ala His Gly
185 190 195

Phe

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGCAAGCTTC ACCATGGACC TCCGAGCAGG GGACTC 36

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 64 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AGCGGGGCCG CACTACTTGC ATCGTCGCGT CCTTGTACTC GAAGCCATGA GCCCCCAGGT 60

GCAG 64

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 199 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION: 1..178

(ix) FEATURE:
(A) NAME/KEY: Region
(B) LOCATION: -21..0

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Met Asn Cys Val Cys Arg Leu Val Leu Val Val Leu Ser Leu Trp Pro
    -20                 -15                 -10

Asp Thr Ala Val Ala Pro Gly Pro Pro Pro Gly Pro Pro Arg Val Ser
-5                  1                   5                   10

Pro Asp Pro Arg Ala Glu Leu Asp Ser Thr Val Leu Leu Thr Arg Ser
            15                  20                  25

Leu Leu Ala Asp Thr Arg Gln Leu Ala Ala Gln Leu Arg Asp Lys Phe
        30                  35                  40

Pro Ala Asp Gly Asp His Asn Leu Asp Ser Leu Pro Thr Leu Ala Met
    45                  50                  55

Ser Ala Gly Ala Leu Gly Ala Leu Gln Leu Pro Gly Val Leu Thr Arg
60                  65                  70                  75

Leu Arg Ala Asp Leu Leu Ser Tyr Leu Arg His Val Gln Trp Leu Arg
                80                  85                  90

Arg Ala Gly Gly Ser Ser Leu Lys Thr Leu Glu Pro Glu Leu Gly Thr
            95                  100                 105

Leu Gln Ala Arg Leu Asp Arg Leu Leu Arg Arg Leu Gln Leu Leu Met
        110                 115                 120

Ser Arg Leu Ala Leu Pro Gln Pro Pro Pro Asp Pro Pro Ala Pro Pro
    125                 130                 135

Leu Ala Pro Pro Ser Ser Ala Trp Gly Gly Ile Arg Ala Ala His Ala
140                 145                 150                 155

Ile Leu Gly Gly Leu His Leu Thr Leu Asp Trp Ala Val Arg Gly Leu
                160                 165                 170

Leu Leu Leu Lys Thr Arg Leu
            175
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 212 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION: 1..182

(ix) FEATURE:
(A) NAME/KEY: Region
(B) LOCATION: -30..0

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Met Asn Ser Phe Ser Thr Ser Ala Phe Gly Pro Val Ala Phe Ser Leu
-30                 -25                 -20                 -15

Gly Leu Leu Leu Val Leu Pro Ala Ala Phe Pro Ala Pro Val Pro Pro
            -10                 -5                  1

Gly Glu Asp Ser Lys Asp Val Ala Ala Pro His Arg Gln Pro Leu Thr
        5                   10                  15

Ser Ser Glu Arg Ile Asp Lys Gln Ile Arg Tyr Ile Leu Asp Gly Ile
    20                  25                  30

Ser Ala Leu Arg Lys Glu Thr Cys Asn Lys Ser Asn Met Cys Glu Ser
35                  40                  45                  50

Ser Lys Glu Ala Leu Ala Glu Asn Asn Leu Asn Leu Pro Lys Met Ala
                55                  60                  65

Glu Lys Asp Gly Cys Phe Gln Ser Gly Phe Asn Glu Glu Thr Cys Leu
            70                  75                  80

Val Lys Ile Ile Thr Gly Leu Leu Glu Phe Glu Val Tyr Leu Glu Tyr
        85                  90                  95

Leu Gln Asn Arg Phe Glu Ser Ser Glu Glu Gln Ala Arg Ala Val Gln
    100                 105                 110

Met Ser Thr Lys Val Leu Ile Gln Phe Leu Gln Lys Lys Ala Lys Asn
115                 120                 125                 130

Leu Asp Ala Ile Thr Thr Pro Asp Pro Thr Thr Asn Ala Ser Leu Leu
                135                 140                 145

Thr Lys Leu Gln Ala Gln Asn Gln Trp Leu Gln Asp Met Thr Thr His
            150                 155                 160

Leu Ile Leu Arg Ser Phe Lys Glu Phe Leu Gln Ser Ser Leu Arg Ala
        165                 170                 175

Leu Arg Gln Met
    180
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 204 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION: 1..174

(ix) FEATURE:
(A) NAME/KEY: Region
(B) LOCATION: -30..0

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met Ala Gly Pro Ala Thr Gln Ser Pro Met Lys Leu Met Ala Leu Gln
-30 -25 -20 -15

Leu Leu Leu Trp His Ser Ala Leu Trp Thr Val Gln Glu Ala Thr Pro
-10 -5 1

Leu Gly Pro Ala Ser Ser Leu Pro Gln Ser Phe Leu Leu Lys Cys Leu
5 10 15

Glu Gln Val Arg Lys Ile Gln Gly Asp Gly Ala Ala Leu Gln Glu Lys
20 25 30

Leu Cys Ala Thr Tyr Lys Leu Cys His Pro Glu Glu Leu Val Leu Leu
35 40 45 50

Gly His Ser Leu Gly Ile Pro Trp Ala Pro Leu Ser Ser Cys Pro Ser
55 60 65

Gln Ala Leu Gln Leu Ala Gly Cys Leu Ser Gln Leu His Ser Gly Leu
70 75 80

Phe Leu Tyr Gln Gly Leu Leu Gln Ala Leu Glu Gly Ile Ser Pro Glu
85 90 95

Leu Gly Pro Thr Leu Asp Thr Leu Gln Leu Asp Val Ala Asp Phe Ala
100 105 110

Thr Thr Ile Trp Gln Gln Met Glu Glu Leu Gly Met Ala Pro Ala Leu
115 120 125 130

Gln Pro Thr Gln Gly Ala Met Pro Ala Phe Ala Ser Ala Phe Gln Arg
135 140 145

Arg Ala Gly Gly Val Leu Val Ala Ser His Leu Gln Ser Phe Leu Glu
150 155 160

Val Ser Tyr Arg Val Leu Arg His Leu Ala Gln Pro
165 170

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 201 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Ser Arg Arg Glu Gly Ser Leu Glu Asp Pro Gln Thr Asp Ser Ser
1 5 10 15

Val Ser Leu Leu Pro His Leu Glu Ala Lys Ile Arg Gln Thr His Ser
20 25 30

Leu Ala His Leu Leu Thr Lys Tyr Ala Glu Gln Leu Leu Gln Glu Tyr
35 40 45

Val Gln Leu Gln Gly Asp Pro Phe Gly Leu Pro Ser Phe Ser Pro Pro
50 55 60

Arg Leu Pro Val Ala Gly Leu Ser Ala Pro Ala Pro Ser His Ala Gly
65 70 75 80

Leu Pro Val His Glu Arg Leu Arg Leu Asp Ala Ala Ala Leu Ala Ala
85 90 95

Leu Pro Pro Leu Leu Asp Ala Val Cys Arg Arg Gln Ala Glu Leu Asn
100 105 110

Pro Arg Ala Pro Arg Leu Leu Arg Arg Leu Glu Asp Ala Ala Arg Gln
115 120 125

Ala Arg Ala Leu Gly Ala Ala Val Glu Ala Leu Leu Ala Ala Leu Gly
130 135 140

Ala Ala Asn Arg Gly Pro Arg Ala Glu Pro Pro Ala Ala Thr Ala Ser
145 150 155 160

Ala Ala Ser Ala Thr Gly Val Phe Pro Ala Lys Val Leu Gly Leu Arg
165 170 175

Val Cys Gly Leu Tyr Arg Glu Trp Leu Ser Arg Thr Glu Gly Asp Leu
180 185 190

Gly Gln Leu Leu Pro Gly Gly Ser Ala
195 200

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 199 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met Ala Phe Thr Glu His Pro Leu Thr Pro His Arg Arg Asp Leu Cys
1 5 10 15

Ser Arg Ser Ile Trp Leu Ala Arg Lys Ile Arg Ser Asp Leu Thr Ala
20 25 30

Leu Thr Glu Ser Tyr Val Lys His Gln Gly Leu Asn Lys Asn Ile Asn
35 40 45

Leu Asp Ser Ala Asp Gly Met Pro Val Ala Ser Thr Asp Gln Trp Ser
50 55 60

Glu Leu Thr Glu Ala Glu Arg Leu Gln Glu Asn Leu Gln Ala Tyr Arg
65 70 75 80

Thr Phe His Val Leu Leu Ala Arg Leu Leu Glu Asp Gln Gln Val His
85 90 95

Phe Thr Pro Thr Glu Gly Asp Phe His Gln Ala Ile His Thr Leu Leu
100 105 110

Leu Gln Val Ala Ala Phe Ala Tyr Gln Ile Glu Glu Leu Met Ile Leu
115 120 125

Leu Glu Tyr Lys Ile Pro Arg Asn Glu Ala Asp Gly Met Pro Ile Asn
130 135 140

Val Gly Asp Gly Gly Leu Phe Glu Lys Lys Leu Trp Gly Leu Lys Val
145 150 155 160

Leu Gln Glu Leu Ser Gln Trp Thr Val Arg Ser Ile His Asp Leu Arg
165 170 175

Phe Ile Ser Ser His Gln Thr Gly Ile Pro Ala Arg Gly Ser His Tyr
180 185 190

Ile Ala Asn Asn Lys Lys Met
195

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 252 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION: 1..227

(ix) FEATURE:
(A) NAME/KEY: Region
(B) LOCATION: -25..0

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Met Gly Val Leu Leu Thr Gln Arg Thr Leu Leu Ser Leu Val Leu Ala
-25 -20 -15 -10

Leu Leu Phe Pro Ser Met Ala Ser Met Ala Ala Ile Gly Ser Cys Ser
-5 1 5

Lys Glu Tyr Arg Val Leu Leu Gly Gln Leu Gln Lys Gln Thr Asp Leu
10 15 20

Met Gln Asp Thr Ser Arg Leu Leu Asp Pro Tyr Ile Arg Ile Gln Gly
25 30 35

Leu Asp Val Pro Lys Leu Arg Glu His Cys Arg Glu Arg Pro Gly Ala
40 45 50 55

Phe Pro Ser Glu Glu Thr Leu Arg Gly Leu Gly Arg Arg Gly Phe Leu
60 65 70

Gln Thr Leu Asn Ala Thr Leu Gly Cys Val Leu His Arg Leu Ala Asp
75 80 85

Leu Glu Gln Arg Leu Pro Lys Ala Gln Asp Leu Glu Arg Ser Gly Leu
90 95 100

Asn Ile Glu Asp Leu Glu Lys Leu Gln Met Ala Arg Pro Asn Ile Leu
105 110 115

Gly Leu Arg Asn Asn Ile Tyr Cys Met Ala Gln Leu Leu Asp Asn Ser
120 125 130 135

Asp Thr Ala Glu Pro Thr Lys Ala Gly Arg Gly Ala Ser Gln Pro Pro
140 145 150

Thr Pro Thr Pro Ala Ser Asp Ala Phe Gln Arg Lys Leu Glu Gly Cys
155 160 165

Arg Phe Leu His Gly Tyr His Arg Phe Met His Ser Val Gly Arg Val
170 175 180

Phe Ser Lys Trp Gly Glu Ser Pro Asn Arg Ser Arg Arg His Ser Pro
185 190 195

His Gln Ala Leu Arg Lys Gly Val Arg Arg Thr Arg Pro Ser Arg Lys
200 205 210 215

Gly Lys Arg Leu Met Thr Arg Gly Gln Leu Pro Arg
220 225

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 202 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION: 1..180

(ix) FEATURE:
(A) NAME/KEY: Region
(B) LOCATION: -22..0

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Met Lys Val Leu Ala Ala Gly Val Val Pro Leu Leu Leu Val Leu His
-20 -15 -10

Trp Lys His Gly Ala Gly Ser Pro Leu Pro Ile Thr Pro Val Asn Ala
-5 1 5 10

Thr Cys Ala Ile Arg His Pro Cys His Asn Asn Leu Met Asn Gln Ile
15 20 25

Arg Ser Gln Leu Ala Gln Leu Asn Gly Ser Ala Asn Ala Leu Phe Ile
30 35 40

Leu Tyr Tyr Thr Ala Gln Gly Glu Pro Phe Pro Asn Asn Leu Asp Lys
45 50 55

Leu Cys Gly Pro Asn Val Thr Asp Phe Pro Pro Phe His Ala Asn Gly
60 65 70

Thr Glu Lys Ala Lys Leu Val Glu Leu Tyr Arg Ile Val Val Tyr Leu
75 80 85 90

Gly Thr Ser Leu Gly Asn Ile Thr Arg Asp Gln Lys Ile Leu Asn Pro
95 100 105

Ser Ala Leu Ser Leu His Ser Lys Leu Asn Ala Thr Ala Asp Ile Leu
110 115 120

Arg Gly Leu Leu Ser Asn Val Leu Cys Arg Leu Cys Ser Lys Tyr His
125 130 135

Val Gly His Val Asp Val Thr Tyr Gly Pro Asp Thr Ser Gly Lys Asp
140 145 150

Val Phe Gln Lys Lys Lys Leu Gly Cys Gln Leu Leu Gly Lys Tyr Lys
155 160 165 170

Gln Ile Ile Ala Val Leu Ala Gln Ala Phe
175 180

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGCGCTACGG TCGACCCGGC GTTTTTTTTT TTTTTTTTTT TTACG 45

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGAAGGAAAA AAGCGGCCGC TACA 24

I CLAIM:

1. A nucleic acid molecule encoding a polypeptide selected from the group consisting of:

(a) the nucleic acid molecule of SEQ ID NO:1;

(b) the nucleic acid molecule of SEQ ID NO:3;

(c) a nucleic acid molecule encoding the polypeptide of SEQ ID NO:2 or a biologically active fragment thereof;

(d) a nucleic acid molecule that encodes a polypeptide that is at least 70 percent identical to the polypeptide of SEQ ID NO:2;

(e) a nucleic acid molecule that hybridizes under stringent conditions to any of (a)-(d) above; and

(f) a nucleic acid molecule that is the complement of any of (a)-(e) above.

2. A nucleic acid molecule encoding a polypeptide selected from the group consisting of:

(a') the nucleic acid molecule of SEQ ID NO:4;

(b') a nucleic acid molecule encoding the polypeptide of SEQ ID NO:5 or a biologically active fragment thereof;

(c') a nucleic acid molecule that encodes a polypeptide that is at least 70 percent identical to the polypeptide of SEQ ID NO:5;

(d') a nucleic acid molecule that hybridizes under stringent conditions to any of (a')-(c') above; and

(e') a nucleic acid molecule that is the complement of any of (a')-(d') above.

3. The nucleic acid molecule that is SEQ ID NO:1.

4. The nucleic acid molecule that is SEQ ID NO:3.

5. A nucleic acid molecule encoding the polypeptide of SEQ ID NO:2.

6. A nucleic acid molecule encoding amino acids 1-198 of SEQ ID NO:2

7. A vector comprising a nucleic acid molecule of any of claims 1 to 6.

8. A host cell comprising a vector of claim 7.

9. A process for producing an NNT-1 polypeptide comprising the steps of:

(a) expressing a polypeptide encoded by a nucleic acid of any of claims 1-6 in a suitable host; and

(b) isolating the polypeptide.

10. An NNT-1 polypeptide selected from the group consisting of:

(a) the polypeptide of SEQ ID NO:2;

(b) the polypeptide that is amino acids 1-198 of SEQ ID NO:2;

(c) a polypeptide that is at least 70 percent identical to the polypeptide of (a) or (b); and

(d) a biologically active fragment of any of (a)-(c).

11. An NNT-1 polypeptide selected from the group consisting of:

(a') the polypeptide of SEQ ID NO:5;

(b') the polypeptide that is amino acids 1-198 of SEQ ID NO:5;

(c') a polypeptide that is at least 70 percent identical to the polypeptide of (a') or (b'); and

(d') a biologically active fragment of any of (a')-(c').

---

12. An NNT-1 polypeptide that is the polypeptide of SEQ ID NO:2 or a biologically active fragment thereof.

13. An NNT-1 polypeptide that is the polypeptide of SEQ ID NO:5 or a biologically active fragment thereof.

14. The NNT-1 polypeptide of claim 12 or 13 that does not possess an amino terminal methionine.

15. The NNT-1 polypeptide of claim 12 or 13 that additionally possesses an amino terminal methionine.

16. An antibody or fragment thereof which specifically binds human NNT-1. .

17. The antibody of claim 16 that is a monoclonal antibody.

18. A method of treating a patient suffering from a neurological or immunological disease or disorder, comprising administering to said patient an effective amount of an NNT-1 polypeptide according to any of Claims 12-15.

19. A method according to Claim 18, wherein said disease or disorder is selected from Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Charcot-Marie-Tooth syndrome, Huntington's disease, peripheral neuropathy, dystrophy, or degeneration of the neural retina.

20. A method according to Claim 18, wherein said disease or disorder is characterized by a deficiency of B-cells or T cells.

21. A method according to Claim 20, wherein said disease or disorder is common variable immunodeficiency (CVID), selective IgA deficiency, hypogammaglobulinemia, and X-linked aggammaglobulinemia.

22. A method of boosting immunoreactivity and antibody production upon vaccination, comprising administering to a patient in need thereof an effective amount of an NNT-1 polypeptide according to any of Claims 12-15.

23. A method of treating an inflammatory condition in a patient in need thereof, comprising administering to said patient an effective amount of NNT-1 polypeptide according to any of Claims 12-15.

FIG. 1

```
   1  ATTAAAGCTT CGCCGGAGCC GCGGCTCGCC CTCCCACTCC GCCAGCCTCC
  51  GGGAGAGGAG CCGCACCCGG CCGGCCCAGC CCCAGCCCCA TGGACCTCCG
 101  AGCAGGGGAC TCGTGGGGGA TGTTAGCGTG CCTGTGCACG GTGCTCTGGC
 151  ACCTCCCTGC AGTGCCAGCT CTCAATCGCA CAGGGGACCC AGGGCCTGGC
 201  CCCTCCATCC AGAAAACCTA TGACCTCACC CGCTACCTGG AGCACCAACT
 251  CCGCAGCTTG GCTGGGACCT ATCTGAACTA CCTGGGCCCC CCTTTCAACG
 301  AGCCAGACTT CAACCCTCCC CGCCTGGGGG CAGAGACTCT GCCCAGGGCC
 351  ACTGTTGACT TGGAGGTGTG GCGAAGCCTC AATGACAAAC TGCGGCTGAC
 401  CCAGAACTAC GAGGCCTACA GCCACCTTCT GTGTTACTTG CGTGGCCTCA
 451  ACCGTCAGGC TGCCACTGCT GAGCTGCGCC GCAGCCTGGC CCACTTCTGC
 501  ACCAGCCTCC AGGGCCTGCT GGGCAGCATT GCGGGCGTCA TGGCAGCTCT
 551  GGGCTACCCA CTGCCCCAGC CGCTGCCTGG GACTGAACCC ACTTGGACTC
 601  CTGGCCCTGC CCACAGTGAC TTCCTCCAGA AGATGGACGA CTTCTGGCTG
 651  CTGAAGGAGC TGCAGACCTG GCTGTGGCGC TCGGCCAAGG ACTTCAACCG
 701  GCTCAAGAAG AAGATGCAGC CTCCAGCAGC TGCAGTCACC CTGCACCTGG
 751  GGGCTCATGG CTTCTGACTT CTGACCTTCT CCTCTTCGCT CCCCCCC
```

FIG.2

Genomic sequences of the human NNT-1

```
  1  aacctgcgag tgggcctggc ggatgggatt attaaagctt cgccggagcc
 51  gcggctcgcc ctcccactcc gccagcctcc gggagaggag ccgcacccgg
101  ccggcccagc cccagccccA TGGACCTCCG AGCAGgt--- ----------

     -----( >1 kb )----------------------------  tgaaaaccca

151  aactagccct gctcttcata acatgacaag cagcgcccca tctgatacct
201  aaaccgacca agtcacagcc ctccaactca ccctctgcct gcccagacct
251  caccacatcc ttgstggact caaacctcaa ccgcactaaa tcaaccaaat
301  cccaagtcta aactaatctg aaacttttaa agtaacccag tccttaaacc
351  taacctagcc caatgccaat tatatctacc ctagccaaac cctaactgcc
401  tttgccagtc caaagtgtcc actgaatcct caccttggtc ctcactgaaa
451  atcccagaaa agcatatttc cccactgccc acatccctcc ttacagcacc
501  caaccctggc ctctggactc ctggtatcct gggatgtcca aactctgcag
551  tgccatcagc caacaagccc gactcgtcaa atgcacctct ctcccttcct
601  gtccccaccc ttgcaggctg atggaaaggc ctcattgaag tccaactttt
651  ccccacctaa caccaagaac ggggtgaacc tccacactgc caccgttccc
```

FIG.2A

```
 701  tgagagtgag cactaaatct ccttcaatct aaccccaccc tacacttccc
 751  acactcagga atcacatcct agaatatacc caaaactaag ccccataagg
 801  cagcccgacc ctagtggtct aaccctatac cttgcttcct atgggtgagt
 851  ctgttcttgg cggccgcctc tctcctgctt cctcccttag agctgactgt
 901  gctcagcctg ccagctctga catgtgctgt ctcccaccct ctgactcccc
 951  tcaagctgca gtgggactgg aagactggca ggaagctagg gtacaactgg
1001  aacacaggca ggtcgacctg cagtccctag gcctggcccc gtccctccat
1051  gtacacacat atacatgttg gcacacacac agtggcacac atgccaaaga
1101  ctctctcagc tgacacacag atccattctc aagtatctac tgatagacac
1151  tcatgcgtgc caagtcctca tcctcaaaca tacacatgcc tctctttctc
1201  tcccgtcttg ccaggagtgt ttcccctcct ccatcccctc tgcctcccat
1251  ctggtgtccc accctcaccc cccacccagc ccaaggtggg gacagacacc
1301  tgaggggctg ccagctgctt ccccgtgtgg gcccgggccg cgctcatgct
1351  tctcgtccat cctgcccaca gGGGACTCGT GGGGGATGTT AGCGTGCCTG
1401  TGCACGGTGC TCTGGCACCT CCCTGCAGTG CCAGCTCTCA ATCGCACAGG
1451  GGACCCAGGG CCTGGCCCCT CCATCCAGAA AACCTATGAC CTCACCCGCT
1501  ACCTGGAGCA CCAACTCCGC AGCTTGGCTG GGACCTATgt gagtatccag
1551  cgtaggaatc tgggagttgg ggaggagtga ggagttgggg aaagacagtc
1601  ctaaccgtgg agggttctgg taaatgatgg ggtgaggagg ggctctttgg
```

FIG.2B

1651 ctcccaccag tccccctgtc tggtctatct cctgcccttc cctcttaggt

1701 ggcccccccа cttccccatc cctggcccca ggactaggca tgtgggcagg

1751 cctcgcaccc gccttggccc attgccccac tggctgccag cccagccgcc

1801 cgcctccccc tgggggccgg ggaagtctcc tctgtttaca ccgtgttgtg

1851 gtgtctcttg cgcgggcggg gttgggtggg gacagagggg ccccacctcc

1901 catgcctgcg ttccagctcg cctctgcccc cagacctggg gccctgctgc

1951 tctggaccca ggggcctccc ttccgtctgc ctctcccatc ctagctgggc

2001 ctcctagggg ggtcatgggg gaaggggact gtagggaacc caggcagtag

2051 tggcaggggg tttagggtgt ggatggaggt tatgctgtaa ggatttgggg

2101 gtggtccaga ggtgttcaga gagcccagga gagaaggaag gagggttgga

2151 ggagccgagg caccatgggg aaccggcccc ctcttcccgt gttcctcttc

2201 cacatcccag accctactct ggagccaggg aaagaaaagg gaagaaggtg

2251 gcgggggagc tggctccagc cccaggatac accgaggaaa ttagtttgtc

2301 tctgtgcttg tcagcgtgtg aacctccccc tgggcccttg cctatcccag

2351 gcctctcccc ttgcttctcc cttctttccc agttatacat ctccctcatc

2401 cctttccctg ggccccagcc gctcccccga gggttggaaa gggctctgcc

2451 ctcttcccta taccatgctg tcttccatag ccttcctcct gtcctactca

2501 tgagactgcc tccatttctt ccttctgcaa ccctgctcct atcagctgaa

FIG.2C

2551 cccttctttc ggagtgttag tgagtacccg tctctcccca gcccctcagc

2601 tggtgggcct gggtgtgtca gcggcaaatg gggctctggt tccaatgggc

2651 cactctcatc tctctcttgt tccttgtgca gaaaaccttt gcttcactcc

2701 actgccctct ctagttcccg acccttttc tctcctggct ttccctgcca

2751 aatttctcca aggagtggtc tacaccctct gcctccactt cctctccacc

2801 cactcacttc ttaacccccc gcaatctggc ttccaggccc cagcaatggt

2851 tctctccaag gtcgtcaggc acctccttgc caagcccgac agtgttttga

2901 aggctcattc tccttgctgt ctgttttgca gccacactgc tgagcgctgc

2951 tgccttctcg aactcctctt ccttggtctc tgcactctcc tgggccacct

3001 tctacctctc cagctcctcc aggctcctct tcctctctgt cctgccccca

3051 cagcgggcac tctcccaagg tttgcccacc cagccaatca gcacgtcctt

3101 cctgagcgtc ttgtgcgtct cctcctcctc ctttttctac gcctctccat

3151 tggagagctc accaccgcca ctgcttcaac tgtcacctgc atacaaatga

3201 tatccttatt ggaaaaactc agggaggcca tgaacaaaga agcctagcat

3251 ggagacaggg ccagtgtcag gggacacaaa aaatagaaac tttgggagca

3301 ggtatctcct tggtggtgag ccagcggctc tgccctcctc cttccccatc

3351 accctctcct tttcacagCT GAACTACCTG GGCCCCCCTT TCAACGAGCC

3401 AGACTTCAAC CCTCCCCGCC TGGGGGCAGA GACTCTGCCC AGGGCCACTG

3451 TTGACTTGGA GGTGTGGCGA AGCCTCAATG ACAAACTGCG GCTGACCCAG

FIG.2D

3501 AACTACGAGG CCTACAGCCA CCTTCTGTGT TACTTGCGTG GCCTCAACCG

3551 TCAGGCTGCC ACTGCTGAGC TGCGCCGCAG CCTGGCCCAC TTCTGCACCA

3601 GCCTCCAGGG CCTGCTGGGC AGCATTGCGG GCGTCATGGC AGCTCTGGGC

3651 TACCCACTGC CCCAGCCGCT GCCTGGGACT GAACCCACTT GGACTCCTGG

3701 CCCTGCCCAC AGTGACTTCC TCCAGAAGAT GGACGACTTC TGGCTGCTGA

3751 AGGAGCTGCA GACCTGGCTG TGGCGCTCGG CCAAGGACTT CAACCGGCTC

3801 AAGAAGAAGA TGCAGCCTCC AGCAGCTGCA GTCACCCTGC ACCTGGGGGC

3851 TCATGGCTTC tgacttctga ccttctcctc ttcgctcccc cttcaaaccc

3901 tgctcccact ttgtgagagc cagccctgta tgccaacacc tgttgagcca

3951 ggagacagaa gctgtgagcc tctggccctt tcctggaccg gctgggcgtg

4001 tgatgcgatc agccctgtct cctccccacc tcccaaaggt ctaccgagct

4051 ggggaggagg tacagtaggc cctgtcctgt cctgtttcta caggaagtca

4101 tgctcgaggg agtgtgaagt ggttcaggtt ggtgcagagg cgctcatggc

4151 ctcctgcttc ttgcctacca cttggccagt gcccacccag cccctcaggt

4201 ggcacatctg gagggcaggg gttgaggggc caccaccaca catgcctttc

4251 tggggtgaag ccctttggct gccccactct ccttggatgg gtgttgctcc

FIG.2E

```
4301  cttatcccca aatcactcta tacatccaat tcaggaaaca aacatggtgg
4351  caattctaca caaaaagaga tgagattaac agtgcagggt tggggtctgc
4401  attggaggtg ccctataaac cagaagagaa aatactgaaa gcacaggggc
4451  agggacagac cagaccagac ccaggagtct ccaaagcaca gagtggcaaa
4501  caaaacccga gctgagcatc aggaccttgc ctcgaattgt cttccagtat
4551  tacggtgcct cttctctgcc ccctttccca gggtatctgt gggttgccag
4601  gctggggagg gcaaccatag ccacaccaca ggatttcctg aaagtttaca
4651  atgcagtagc attttggggt gtagggtggc agctccccaa ggccctgccc
4701  cccagcccca cccactcatg actctaagtg tgttgtatta atatttattt
4751  atttggagat gttatttatt agatgatatt tattgcagaa tttctattct
4801  tgtattaaca aataaaatgc ttgccccaga acttagtctc tttgcccagc
4851  ctcacccctc ctggtgctca tcagactctt gccacccctg gctcccactc
4901  cctgcttgcc tctggtggag ctgcacagag ctctgggaag aggccctctt
4951  cctccccgca ctggggcgat gggcgcacct cagacttacc cactgctgct
5001  gccaccacca acccccttgat ccctcagtcc tcccacacag cttctgtcca
5051  ccccaggttt ccctcacccc acctttgcta agtcttcctc a
```

FIG.3

```
-27                                  1
 MDLR AGDSWGMLAC LCTVLWHLPA VPALNRTGDP GPGPSIQKTY   17

DLTRYLEHQL RSLAGTYLNY LGPPFNEPDF NPPRLGAETL PRATVDLEVW   67

RSLNDKLRLT QNYEAYSHLL CYLRGLNRQA ATAELRRSLA HFCTSLQGLL  117

GSIAGVMAAL GYPLPQPLPG TEPTWTPGPA HSDFLQKMDD FWLLKELQTW  167

                                   198
LWRSAKDFNR LKKKMQPPAA AVTLHLGAHG F*                     198
```

FIG.4

```
  1  TATTATTAAA GCTTCGCCGG AGCCGCGGCT CGCCCTCCCA CTCCGCCAGC
 51  CTCTGGGAGA GGAGCCGCGC CCGGCCGGCC CGGCCCCCAG CCCCATGGAC
101  CTCCGAGCAG GGGACTCGTG GGGGATGTTA GCTTGCCTAT GCACGGTGCT
151  GTGGCACCTC CCTGCAGTGC CAGCTCTTAA TCGCACAGGA GATCCAGGCC
201  CTGGCCCCTC CATCCAGAAA ACCTATGACC TCACCCGCTA CCTGGAGCAT
251  CAACTCCGCA GCTTAGCTGG GACCTACCTG AACTACCTGG GGCCCCCTTT
301  CAACGAGCCT GACTTCAATC CTCCTCGACT GGGGGCAGAA ACTCTGCCCA
351  GGGCCACGGT CAACTTGGAA GTGTGGCGAA GCCTCAATGA CAGGCTGCGG
401  CTGACCCAGA ACTATGAGGC GTACAGTCAC CTCCTGTGTT ACTTGCGTGG
451  CCTCAACCGT CAGGCTGCCA CAGCTGAACT CCGACGTAGC CTGGCCCACT
501  TCTGTACCAG CCTCCAGGGC CTGCTGGGCA GCATTGCAGG TGTCATGGCG
551  ACGCTTGGCT ACCCACTGCC CCAGCCTCTG CCAGGGACTG AGCCAGCCTG
601  GGCCCCTGGC CCTGCCCACA GTGACTTCCT CCAGAAGATG GATGACTTCT
651  GGCTGCTGAA GGAGCTGCAG ACCTGGCTAT GGCGTTCAGC CAAGGACTTC
701  AACCGGCTTA AGAAGAAGAT GCAGCCTCCA GCAGCTTCAG TCACCCTGCA
751  CTTGGAGGCA CATGGTTTCT GACCTCTGAC CCTTAACCCC CACACCTCCA
801  GGCCCAGTCA GCTGTGCTT
```

FIG.5

```
-27                                1
 MDLRAGDSWG MLACLCTVLW HLPAVPALNR TGDPGPGPSI QKTYDLTRYL   23

 EHQLRSLAGT YLNYLGPPFN EPDFNPPRLG AETLPRATVN LEVWRSLNDR   73

 LRLTQNYEAY SHLLCYLRGL NRQAATAELR RSLAHFCTSL QGLLGSIAGV  123

 MATLGYPLPQ PLPGTEPAWA PGPAHSDFLQ KMDDFWLLKE LQTWLWRSAK  173

                           198
 DFNRLKKKMQ PPAASVTLHL EAHGF*                            198
```

FIG.6

```
                1                                                  50
        NNT-1   ..........  ..........  .       MDL RAGDSWGMLA CLCTVLWHLP
        Il-11   ..........  ..........  ..........  MNCVCRLVLV VLS..LWPDT
         Il-6                          MNSFSTSAF GPVAFSLGLL LVLPAAFPAP
         GCSF   ..........  ..........  ....MAGPAT QSPMKLMALQ LLL...WHSA
Cardiotrophin   ..........  ..........  ..........  ....           MSRREG
         CNTF   ..........  ..........  ..........  ..........  ....MAFTEH
   Oncostatin   ..........  ..........  ..........  ..MGVLLTQR TLLSLVLALL
          LIF   ..........                          MKVLAAGVVP LLLVLHWKHG

                51                                                 100
        NNT-1   AVPALNRTG.  ...DPGPGPS IQKTYDLTRY LEHQLRSLAG TYLNYLGPPF
        Il-11   AVAPGPPPGP  PRVSPDPRAE LDSTVLLTRS LLADTRQLAA QLRDKFPA..
         Il-6   VPPGEDSKDV  AAPHRQPLTS SERIDKQIRY ILDGISALRK ETCN......
         GCSF   LWTVQEATPL  GPASSLPQSF .LLKCLEQVRK IQGDGAALQE KLCA......
Cardiotrophin   SLEDPQTDSS  VSLLPHLEAK IRQTHSLAHL LTKYAEQLLQ EYVQLQGDPF
         CNTF   S.........  .PLTPHRRDL CSRSIWLARK IRSDLTALTE SYVKHQGLNK
   Oncostatin   FPSMASMAAI  GSCSKEYRVL LGQLQKQTD. LMQDTSRLLD PYIRIQGLDV
          LIF   AGSPLPITPV  NATCAIRHPC HNNLMNQIRS QLAQLNGSAN AL........

                101                                                150
        NNT-1   NEPDFNPPRL  GAETLPRATV DLEVWRSLND KLRLTQN..Y EAY.SHLLCY
        Il-11   .DGDHNLDSL  PTLAMSAGAL GALQLPGVLT RLR.......  ....ADLLSY
         Il-6   ...KSNMCES  SKEALAENNL NLPKMAEKDG CFQSGFN..E ETCLVKIITG
         GCSF   ...TYKLCHP  EELVLLGHSL GIPW.APLSS CPSQALQ..L AGCLSQLHSG
Cardiotrophin   ...GLPSFSP  PRLPVAGLSA PAPSHAGLPV HERLRLD..A AALAALPPLL
         CNTF   ...NINLDSA  DGMPVAS...  .TDQWSELTE AERLQEN..L QAYRTFHVLL
   Oncostatin   PKLREHCRER  PGAFPSEETL RGLGRRGFLQ TLNATLGCVL HRLADLEQRL
          LIF   ....FILYYT  AQGEPFPNNL DKLCGPNVTD FPPFHANGTE KAKLVELYRI

                151                                                200
        NNT-1   LRGLN..RQA  ATAELR...R SLAHFCTSLQ GLLGSIAGVM AAL..GYP.L
        Il-11   LRHVQWLRRA  GGSSLKTLEP ELGTLQARLD RLLRRLQLLM SRL..ALP.Q
         Il-6   LLEFEVYLEY  LQNRFESSEE QARAVQMSTK VLIQFLQKKA KNL..DAI.T
         GCSF   LFLYQGLLQA  LEGISPELGP TLDTLQLDVA DFATTIWQQM EEL..GMA.P
Cardiotrophin   D.AVCRRQAE  LNPRAPRLLR RLEDAARQAR ALGAAVEALL AAL..GAANR
         CNTF   ARLLEDQQVH  FTPTEGDFHQ AIHTLLLQVA AFAYQIEELM ILL..E..YK
   Oncostatin   PKAQDLERSG  LNIEDLEKLQ MARPNILGLR NNIYCMAQLL DNS..DTAEP
          LIF   VVYLGTSLGN  ITRDQKILNP SALSLHSKLN ATADILRGLL SNVLCRLCSK
```

FIG.6A

```
                201                                                 250
      NNT-1     PQPLPGTEPT WTPGPAHSDF LQKMDDFWLL KELQTWLWRS AKDFNR..LK
      Il-11     PPPDPPAPPL APPSSAWGGI ...RAAHAIL GGLHLTLDWA VRGLLL..LK
       Il-6     TPDPTTNASL LTKLQAQNQW LQDMTTHLIL RSFKEFLQSS LRALRQ..M*
       GCSF     ALQPTQGA.. MPAFASAFQR RAG..GVLVA SHLQSFLEVS YRVLRH..LA
Cardiotrophin   GPRAEPPAAT ASAASATGVF PAKVLGLRVC GLYREWLSRT EGDLGQ..LL
       CNTF     IPRNEADGMP INVGDG.GLF EKKLWGLKVL QELSQWTVRS IHDLRF..IS
  Oncostatin    TKAGRGASQP PTPTPASDAF QRKLEGCRFL HGYHRFMHSV GRVFSK..WG
        LIF     YHVGHVDVTY GPDTSGKDVF QKKKLGCQLL GKYKQIIAVL AQAF*

                251                                                 300
      NNT-1     KKMQPPAAAV TLHLGAHGF*  ..........  ..........
      Il-11     TRL*.....  ..........  ..........  ..........  ..........
       Il-6
       GCSF     QP*.....  ..........  ..........  ..........  ..........
Cardiotrophin   .PGGSA*
       CNTF     .SHQTGIPAR GSHYIANNKK M*..  ..........  ..........
  Oncostatin    ESPNRSRRHS PHQALRKGVR RTRPSRKGKR LMTRGQLPR*.  ..........
        LIF
```

FIG.7

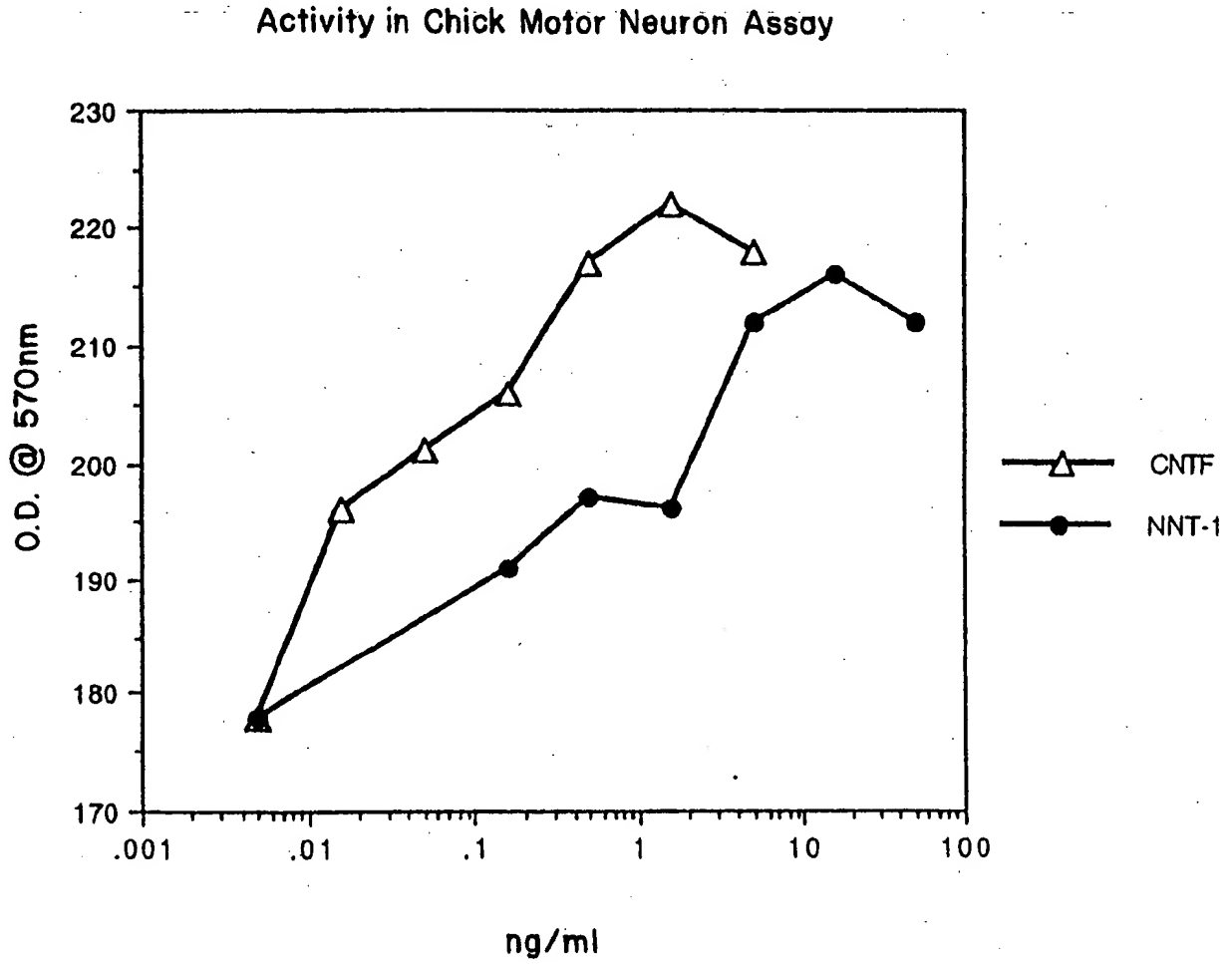

Activity in Chick Motor Neuron Assay
O.D. @ 570nm
230
220
210
200
190
180
170
.001
.01
.1
1
10
100
ng/ml
CNTF
NNT-1

FIG.8

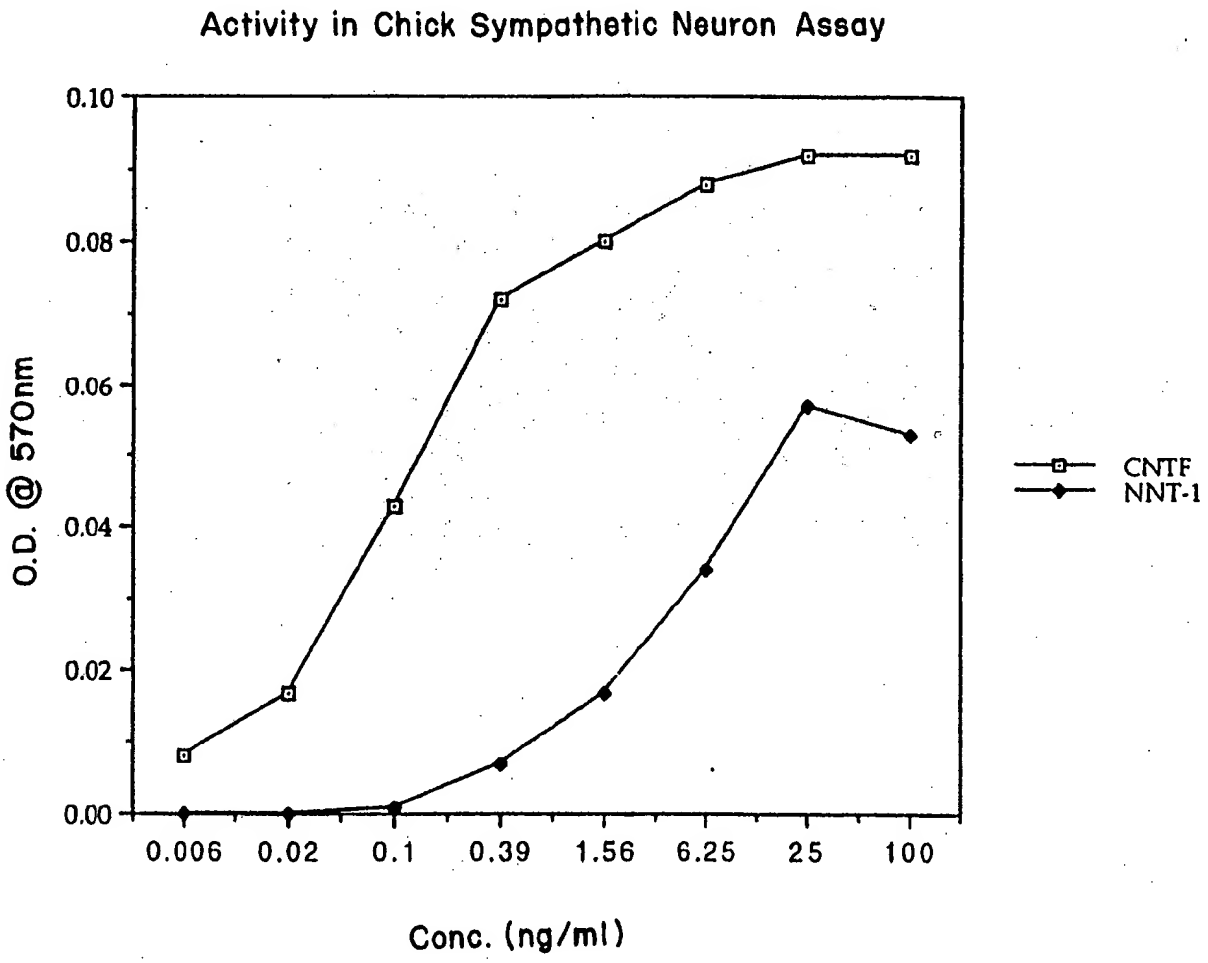

Activity in Chick Sympathetic Neuron Assay
0.10
0.08
0.06
0.04
0.02
0.00
O.D. @ 570nm
0.006
0.02
0.1
0.39
1.56
6.25
25
100
Conc. (ng/ml)
CNTF
NNT-1

FIG. 9

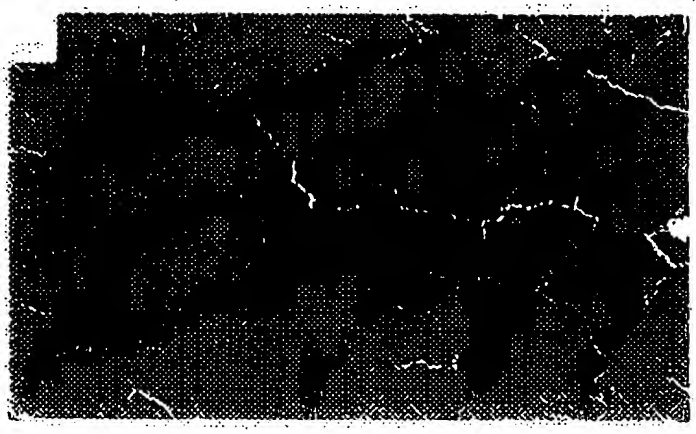


FIG. 10

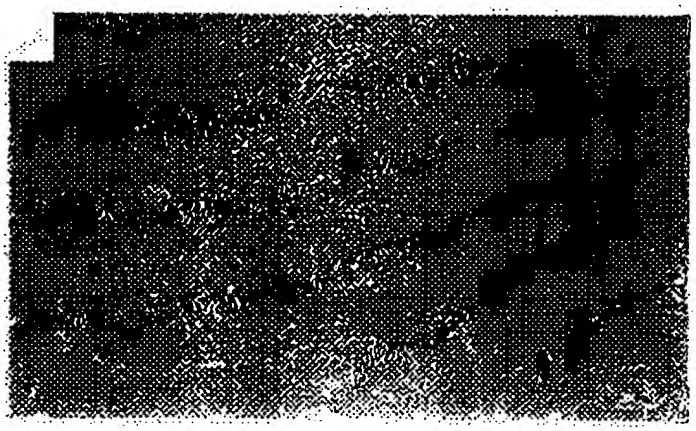


FIG.11

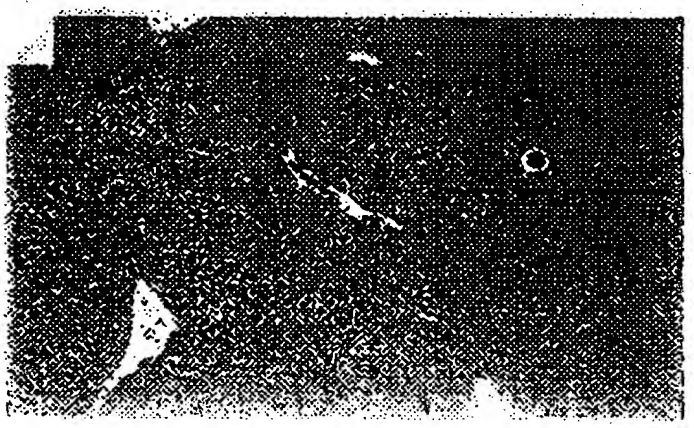


FIG.12

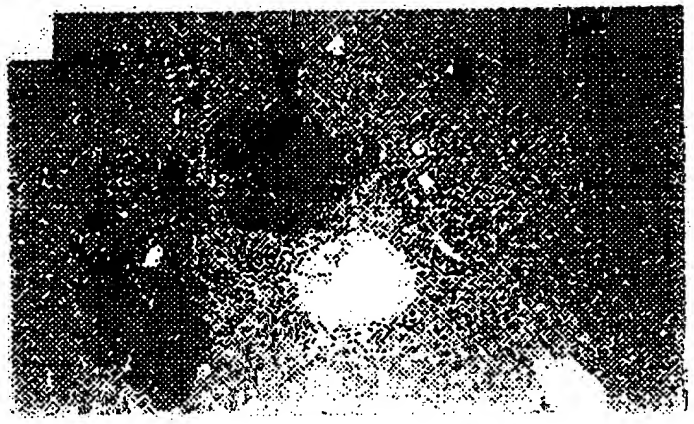


FIG. 13

Serum SAA

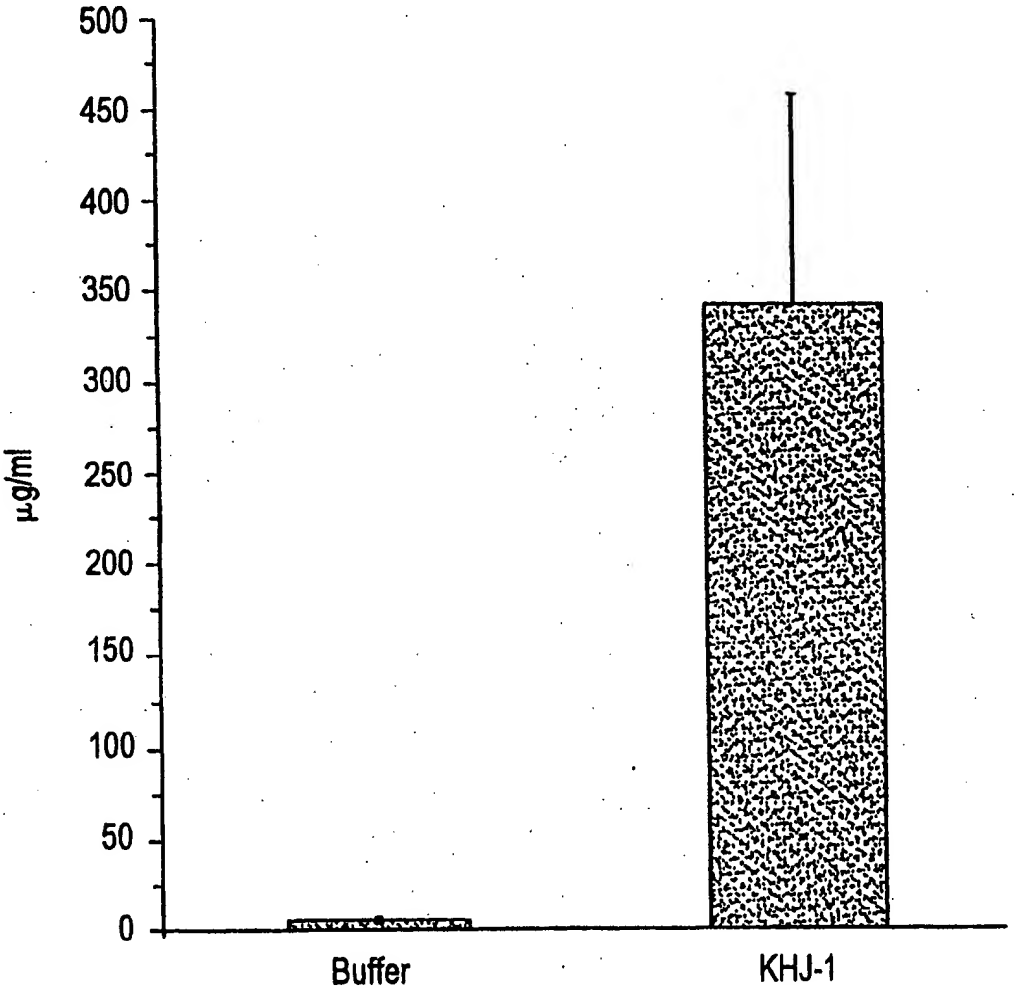


FIG. 14

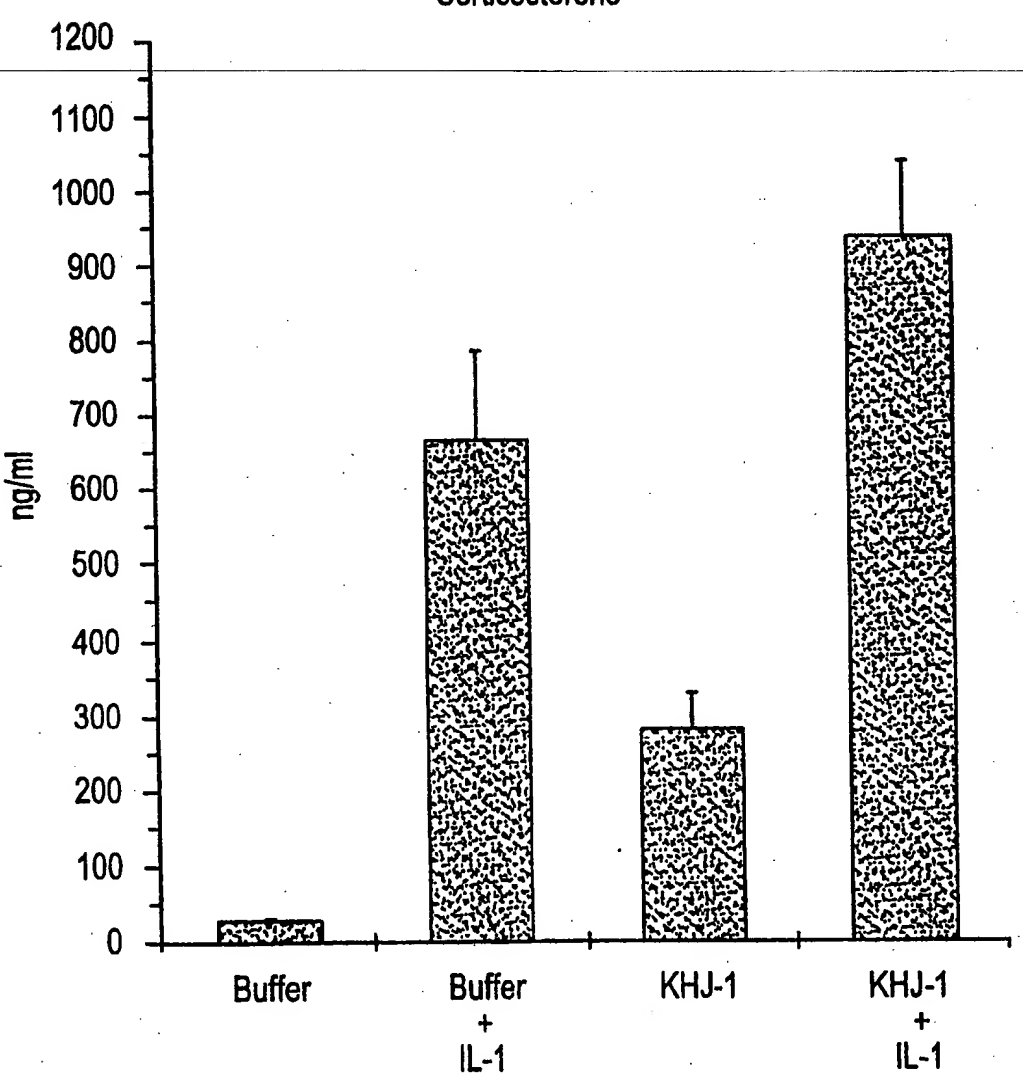

Corticosterone
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
ng/ml
Buffer
Buffer + IL-1
KHJ-1
KHJ-1 + IL-1

FIG. 15

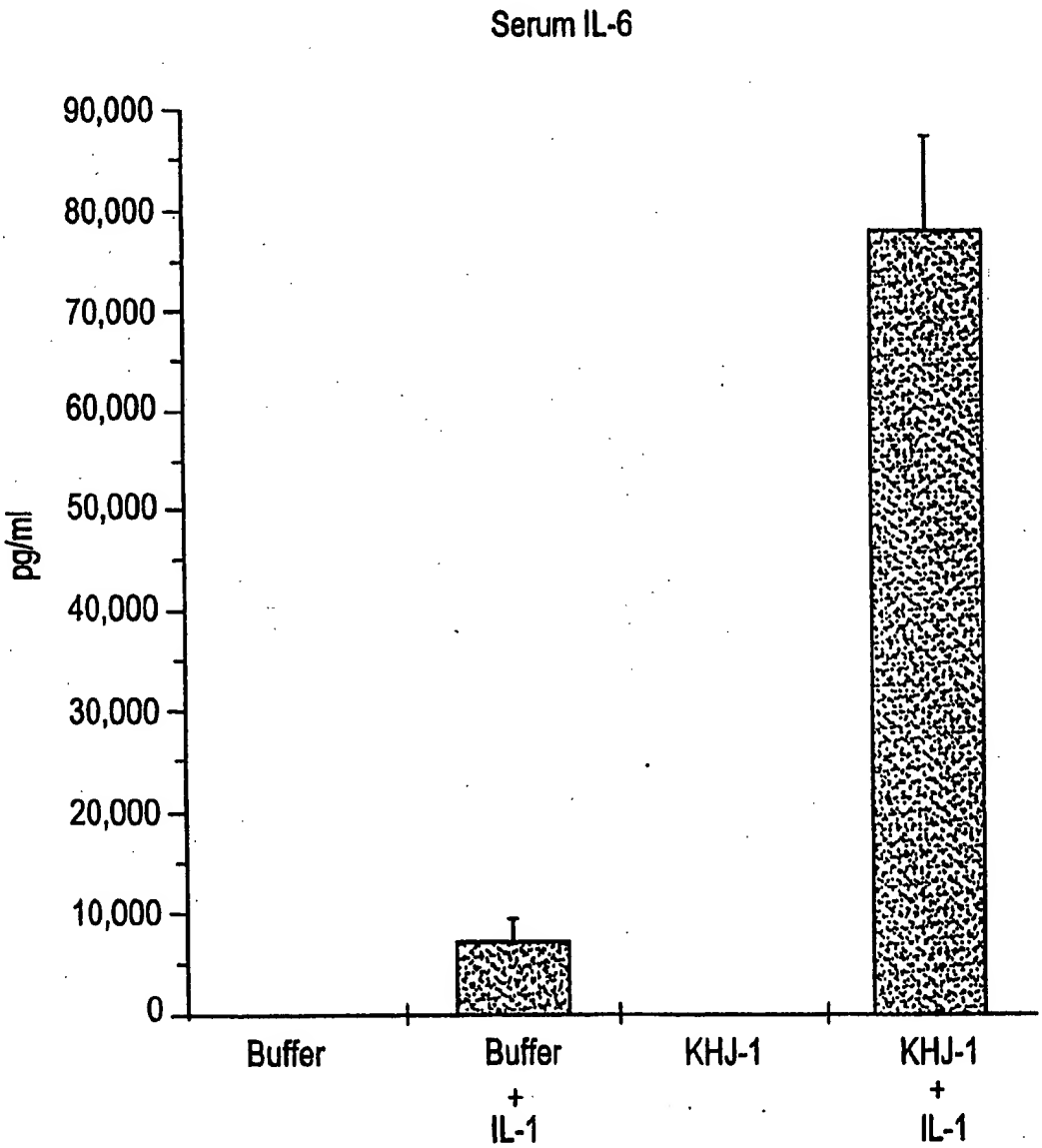

Serum IL-6
90,000
80,000
70,000
60,000
50,000
40,000
30,000
20,000
10,000
0
pg/ml
Buffer
Buffer + IL-1
KHJ-1
KHJ-1 + IL-1

FIG. 16

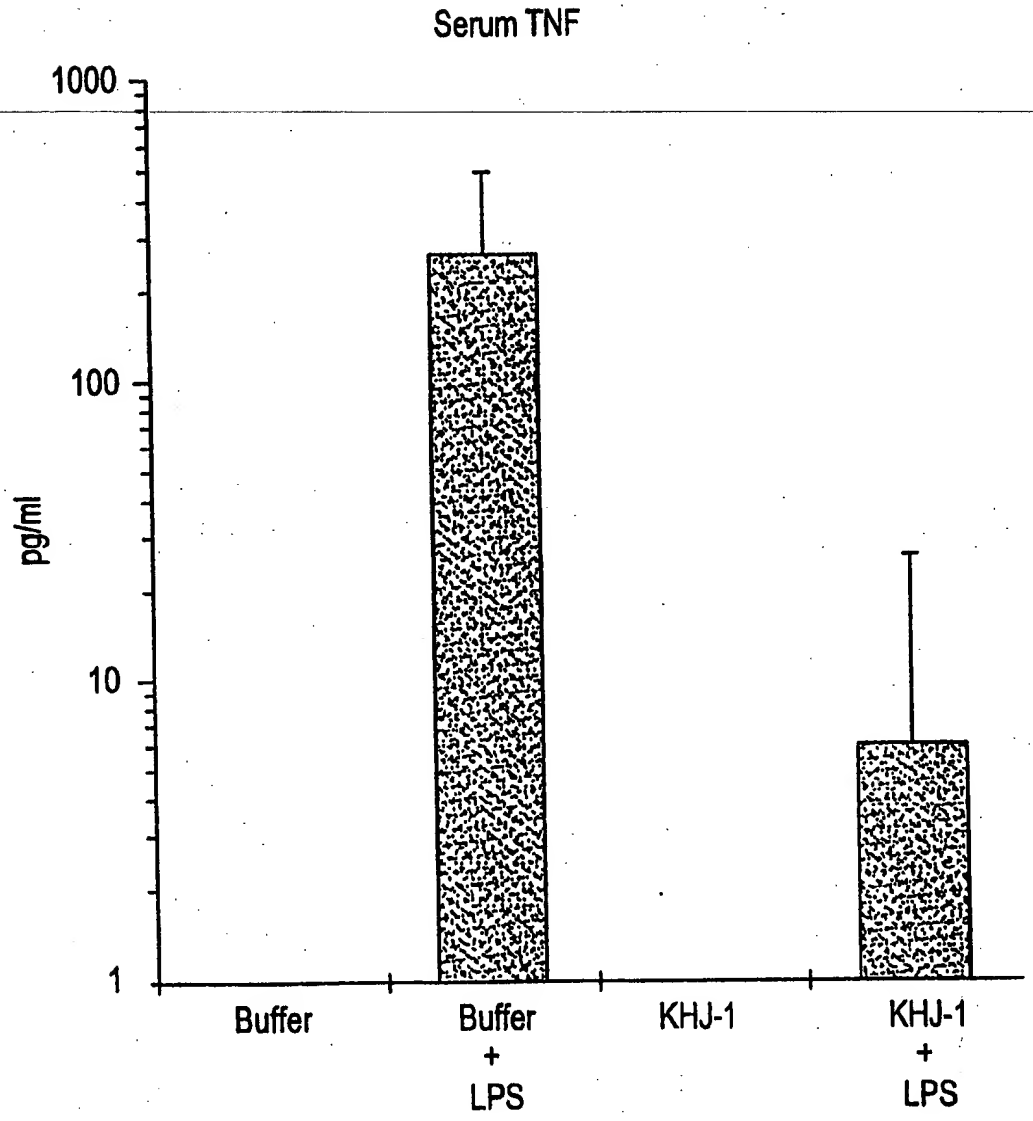


FIG. 17A

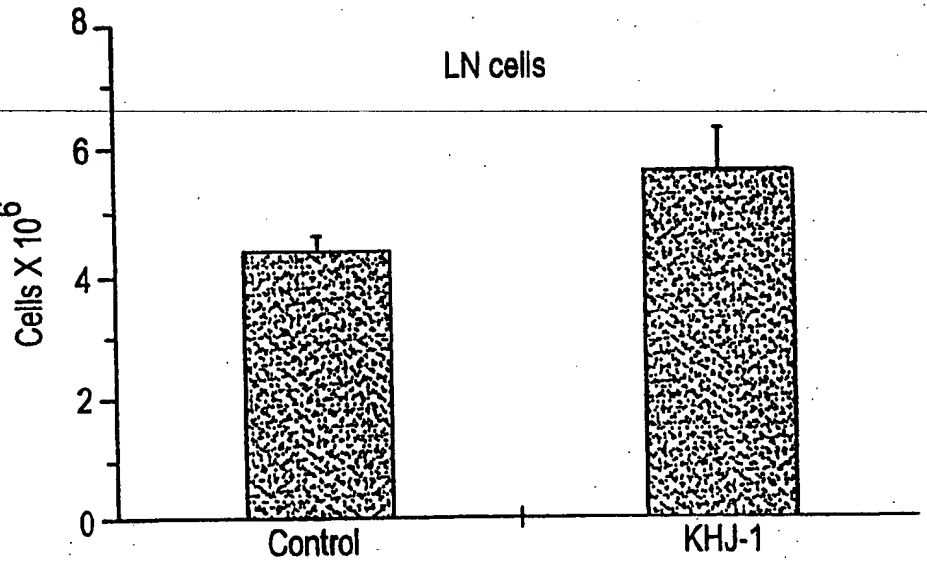
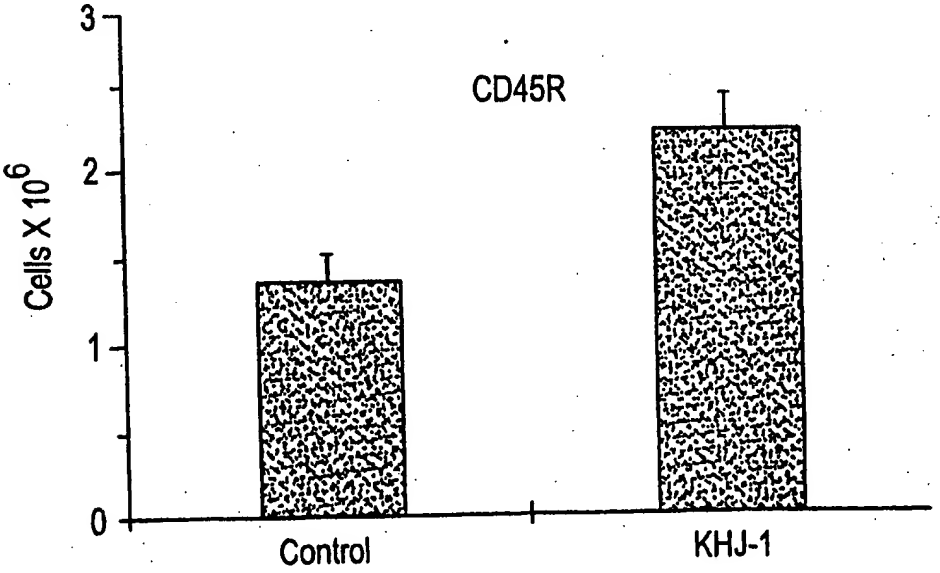


FIG. 17B



INTERNATIONAL SEARCH REPORT

Intern ıal Application No
PCT/US 98/02363

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/18 C07K14/475 C07K16/22 C12N1/21 A61K38/18 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HUMPEL C ET AL: "Monitoring release of neurotrophic activity in the brains of awake rats." SCIENCE, JUL 28 1995, 269 (5223) P552-4, XP002068930 cited in the application see the whole document ---	1,2,10, 11
A	EMBLdatabase Accession number AA015243 03-AUG-1996 (Rel. 48, Created) Marra m et al. XP002068940 see the whole document --- -/--	1,2,10, 11

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 June 1998	09.07.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gurdjian, D

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INTERNATIONAL SEARCH REPORT

Interr. nal Application No
PCT/US 98/02363

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BENIGNI, FABIO ET AL: "Six different cytokines that share GP130 as a receptor subunit, induce serum amyloid A and potentiate the induction of interleukin-6 and the activation of the hypothalamus-pituitary-adrenal axis by interleukin-1" BLOOD (1996), 87(5), 1851-4 CODEN: BLOOAW;ISSN: 0006-4971, XP002068931 see the whole document -----	1,2,10, 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/02363

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 18-23 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.